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Design and Implementation of Environmental DNA Metabarcoding Methods for  
Monitoring the Southern California Marine Protected Area Network

A dissertation submitted in partial satisfaction of the  
requirements for the degree Doctor of Philosophy  
in Biology

by

Zachary Jacob Gold

2020

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## ABSTRACT OF THE DISSERTATION

### Design and Implementation of Environmental DNA Metabarcoding Methods for Monitoring the Southern California Marine Protected Area Network

by

Zachary Jacob Gold

Doctor of Philosophy in Biology

University of California, Los Angeles, 2020

Professor Paul Barber, Chair

Marine protected areas (MPAs) are important tools for maintaining biodiversity and abundance of marine species. However, key to the effectiveness of MPAs is monitoring of marine communities. Current monitoring methods rely heavily on SCUBA-based visual observations that are costly and time consuming, limiting the scope of MPA monitoring. Environmental DNA (eDNA) metabarcoding is a promising cost effective, rapid, and automatable alternative for marine ecosystem monitoring. However, as a developing tool, the utility of eDNA metabarcoding requires improved bioinformatic techniques and reference barcode databases. Furthermore, it is important to understand how eDNA metabarcoding performs relative to visual surveys to better understand the strengths and limitations of each approach. This thesis improves eDNA metabarcoding approaches to survey the nearshore rocky reef and kelp forest ecosystems within the Southern California MPA network. It then tests the effectiveness of eDNA metabarcoding against visual surveys conducted by the Channel Islands National Park Service



Kelp Forest Monitoring Program and Reef Check California. In Chapter 1, I develop FishCARD, a *12S* reference barcode database specific to fishes of the California Current ecosystem. FishCARD improves eDNA metabarcoding taxonomic assignments, resulting in the identification of a broader array of marine vertebrate diversity, including invasive, endangered, and mobile species frequently missed by visual surveys. In Chapter 2, I compare eDNA metabarcoding and visual underwater survey methods inside, on the edge of, and outside the Scorpion State Marine Reserve off Santa Cruz Island. We demonstrate that eDNA captures a broader range of fish taxa than visual surveys and detects fine-scale spatial differences in fish communities. In Chapter 3, I demonstrate that eDNA metabarcoding and visual underwater surveys capture similar biogeographic patterns of fish communities across 44 sites within the Southern California Bight. Importantly, eDNA methods distinguished fish communities inside and outside of Southern California MPAs, finding a greater abundance of target species inside MPAs matching patterns observed through visual surveys. These results built off the collaborative development of the *Anacapa Toolkit* metabarcoding pipeline. Together I demonstrate the utility of eDNA metabarcoding for monitoring MPAs, providing an important complementary tool to visual methods, helping expand MPA monitoring across space, time, and depth.

The dissertation of Zachary Jacob Gold is approved.

Peggy Fong

Richard F. Ambrose

Thomas B. Smith

Paul H. Barber, Committee Chair

University of California, Los Angeles,

2020

*In memory of our heroes taken from us far too soon  
who did their part to make the world a brighter place while protecting and loving our oceans*

*Jonathan Gold,*

*David Klein,*

*Marybeth Guiney, Charles McIlvan, & the 32 other divers onboard the MV Conception*

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## LIST OF ACRONYMS

Acronym	Description
<i>12S</i>	Small subunit gene of the mitochondrial ribosome
<i>16S</i>	Small subunit gene of the mitochondrial ribosome
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variant
betadisper	Multivariate analogue of Levene's test for homogeneity of variances
BLAST	Basic Local Alignment Search Tool
CalCOFI	California Cooperative Oceanic Fisheries Investigation
CAP Analysis	Constrained Analysis of Principle components Analysis
<i>COI</i>	Cytochrome C Oxidase subunit 1 gene of mitochondrial DNA
<i>CRUX</i>	<b>C</b> reating <b>R</b> eference libraries <b>U</b> sing <b>eX</b> isting tools software
<i>CytB</i>	Cytochrome B gene of mitochondrial DNA
DNA	Deoxyribonucleic acid
eDNA	environmental DNA
FishCARD	California fish specific <i>12S</i> California Reference Barcode Database
GPS	Global Positioning System
MPA	Marine Protected Area
NCBI	National Center for Biotechnology Information
NMDS	Non-metric Multidimensional Scaling
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Analysis of Variance
PISCO	Partnership for Interdisciplinary Studies of Coastal Oceans
RNA	Ribonucleic acid
RV	Research Vessel
SCUBA	Self-Contained Underwater Breathing Apparatus
SIO	Scripps Institution of Oceanography
UCLA	University of California, Los Angeles

## GLOSSARY

Term	Description
Amplicon	short DNA fragment generated from metabarcoding
Amplicon Sequence Variant	unique DNA sequence recovered from high-throughput metabarcoding inferred as having a true biological origin
Barcode Region	specific gene locus within the genome
Biological Replicate	a test performed on biologically distinct samples representing an independent sampling event, i.e. three 1 L water samples collected from the same kelp forest habitat
Decontamination	a bioinformatic process to remove non-target sequences inadvertently recovered in the processing of field or laboratory samples
DNA Barcode	short DNA fragment used for species identification
eDNA	a technique based on isolation and sequencing of freely associated DNA from soil or water samples
Lowest Common Ancestor Taxonomic Assignment	species classification based on the deepest shared taxonomic identification between the best aligned DNA reference barcodes
Metabarcoding	a process in which multiple species are identified from bulk DNA (e.g., homogenized gut contents or settlement tile scrapings) or environmental DNA (eDNA) samples (e.g., water and soil) typically by PCR amplification and sequencing of a target gene
Reference Barcode Database	a collection of reference barcode sequences used to assign taxonomy to metabarcoding derived DNA sequences
Reference Barcode Sequence	DNA identification sequence obtained from reference voucher specimen of known origin and identity
Site Occupancy Model	a probabilistic model used to determine the true presence or absence of a species at a site given accounting for imperfect survey detection
Technical Replicate	a test performed on the same sample multiple times, i.e. three PCR reactions using the same extracted DNA sample

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### SUPPLEMENTAL SPREADSHEETS

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Supplemental Spreadsheet 2. Chapter 2 Supplemental Tables Spreadsheet (File Format .xlsx)

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### SUPPLEMENTAL ONLINE MATERIALS

Supplemental Online Material 1. FishCARD Website. Available at

<https://github.com/zjgold/FishCARD>

Supplemental Online Material 2. Supplemental Chapter 1 Supplemental Tables and Appendices

1-6. Available at <https://doi.org/10.1111/2041-210X.13214>

Supplemental Online Material 3. *Anacapa Toolkit* Website. Available at

<https://github.com/limey-bean/Anacapa>

Supplemental Online Material 4. *Anacapa Toolkit* sequence data. Available at

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## BIOGRAPHICAL SKETCH

### Previous Degrees Awarded

Stanford University — B.S. in Marine Biology with Honors and a Minor in Environmental Engineering. Awarded 2015

### Professional Experience

California State Park Ocean Lifeguard, Venture and Monterey, CA, Summers of 2011-2013

### Awards and Honors

#### *Graduate*

- 2020            UCLA IoES Leadership in Sustainability Certificate
- 2019            NSF Graduate Research Internship Project Award
- 2019            Outstanding Grad. Poster, UCLA EEB Research Symposium, 3rd prize
- 2018            UCLA EEB Special Faculty Award for Departmental Service
- 2017            Outstanding Grad. Poster, UCLA EEB Research Symposium, 2nd prize.
- 2016            National Geographic Young Explorer Program
- 2015            National Science Foundation Graduate Research Fellowship, UCLA
- 2015            UCLA Graduate Dean's Scholar Award

#### *Undergraduate*

- 2013            NOAA Ernest F. Hollings Undergraduate Scholarship
- 2013, 2014    Moris K. Udall and Stewart L. Udall Scholarship, Honorable Mention
- 2012            Marine Technology Society Undergraduate Scholarship

### Peer Reviewed Manuscripts

1. Gold, Z., & Palumbi, S. R. (2018). Long-term growth rates and effects of bleaching in *Acropora hyacinthus*. *Coral Reefs*, 37(1), 267-277.
2. Curd, E. E., Gold, Z., Kandlikar, G. S., Gomer, J., Ogden, M., O'Connell, T., Pipes, L., Schweizer, T. Rabichow, L., Lin, M., Shi, B., Barber, P.H., Kraft, N., Wayne, R., Meyer, R.

(2019). Anacapa Toolkit: an environmental DNA toolkit for processing multilocus metabarcode datasets. *Methods in Ecology and Evolution*, 10(9), 1469-1475.

3. Kandlikar, G. S., Gold, Z. J., Cowen, M. C., Meyer, R. S., Freise, A. C., Kraft, N. J., Moberg-Parker, J., Sprague, J., Kushner, D. J., Curd, Emily E (2018). ranacapa: An R package and Shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. *F1000Research*, 7.
4. Meyer, R.S., Curd, E.E., Schweizer, T. Gold, Z., Ruiz Ramos, D., Shirazi, S., Kandlikar, G., Kwan, W., Lin, M., Friese, A., Moberg-Parker, J., Ramos, M.M., Shapiro, B., Sexton, J., Pipes, L., Vedrenne, A.G., Mejia, M.P., Aronson, E., Moore, T., Nielsen, R., Lewin, H., Barber, P.H., Wall, J., Kraft, N. Wayne, R. The California Environmental DNA” CALeDNA” Program. *California Agriculture*. *Accepted article to October citizen science issue*.

## **Presentations**

February 19<sup>th</sup>, 2020. Ocean Sciences Meeting, San Diego, California

*Investigating Patterns of Larval Fish Community Dynamics Over the Past Two Decades*

November 1<sup>st</sup>, 2019. Western Society of Naturalists, Ensenada, Baja California, Mexico

*eDNA: Novel Molecular Techniques for Monitoring MPAs*

March 28<sup>th</sup>, 2019. La Kretz Conservation Genomics Workshop, Stunt Ranch Reserve, CA

*eDNA: Novel Molecular Techniques for Monitoring Biodiversity*

May 16<sup>th</sup>, 2018. MPA Technology Workshop, La Jolla, California

*eDNA: Novel Molecular Techniques for Monitoring MPAs*

March 26<sup>th</sup>, 2018. La Kretz Conservation Genomics Workshop, Stunt Ranch Reserve, CA

*eDNA: Novel Molecular Techniques for Monitoring Biodiversity*

October 2-6<sup>th</sup>, 2017. Indo-Pacific Fish Conference, Papeete, Tahiti, French Polynesia

*Spatial Variation of Environmental DNA in Coral Reef Ecosystems*

August 15-18<sup>th</sup>, 2017. UCGCC Conference, Blue Oak Reserve, California

*Developing and Implementing an Aquatic eDNA Methodology*

July 23-28<sup>th</sup>, 2017. Gordon Research Conference Marine Molecular Ecology, HKUST, HK

*Spatial and Temporal Variation of Environmental DNA in Kelp Forest Ecosystems*

## INTRODUCTION

### *Importance of Marine Resources*

Marine resources are a critical component of our modern society, with the world's major marine ecosystems contributing an estimated \$6 trillion/year to global gross domestic product (Hudson 2012) and another \$27 trillion/year in indirect ecosystem services (Costanza et al. 2014). Moreover, seafood provides nutrition and livelihoods to approximately 4.5 billion people worldwide and is essential to global food security (Béné et al. 2015). Within the United States, California has the largest ocean economy, with marine ecosystems contributing \$42.9 billion annually, and directly providing approximately 408,000 jobs in the year (Kildow and Colgan 2005), benefits that are critical to preserve.

Unfortunately, despite the tremendous value of marine ecosystems to human society, these ecosystems are increasingly under threat (Pauly 1998, Scavia et al. 2002, Harley et al. 2006, Worm et al. 2006, Halpern et al. 2008, Diaz and Rosenberg 2008, Doney et al. 2009, 2012, Cheung et al. 2009, Hoegh-Guldberg and Bruno 2010a, 2010b, Estes et al. 2011, Maxwell et al. 2013, Poloczanska et al. 2013). Marine biodiversity is compromised from both local (e.g. overfishing, pollution, habitat loss) and global anthropogenic stressors (Pauly 1998, Scavia et al. 2002, Harley et al. 2006, Worm et al. 2006, Halpern et al. 2008, Diaz and Rosenberg 2008, Doney et al. 2009, 2012, Cheung et al. 2009, Hoegh-Guldberg and Bruno 2010a, 2010b, Estes et al. 2011, Maxwell et al. 2013, Poloczanska et al. 2013). Currently, over 85% of the world's fisheries are over-exploited, depleted, or collapsed, a problem that is growing worse with increasing seafood demand (Ye et al. 2011). Overfishing causes drastic changes in marine ecosystem dynamics including the loss of fish biodiversity and top and meso-predators (Pauly

1998, Bolster, Jeff et al. 2012), resulting in phase shifts towards less complex, less resilient marine ecosystems (Pauly 1998, Bolster, Jeff et al. 2012). Thus overfishing remains a major global threat to marine biodiversity and food security globally (Pauly 1998, Edgar et al. 2007, Bolster, Jeff et al. 2012).

In addition to over-fishing, intensive urbanization of coastal environments is also drastically altering marine ecosystems, leading to severe losses of marine biodiversity and erosion of ecosystem health (Shahidul Islam and Tanaka 2004, Halpern et al. 2008, Diaz and Rosenberg 2008). Urbanization has drastically altered the hydraulic, nutrient, thermal, and erosion processes of coastal landscapes over the past century, leading to marked shifts in local marine biodiversity (Schiel et al. 2004, Ahn et al. 2005, Alberti 2005, Feagin et al. 2005, Sheng and Wilson 2009, Pataki et al. 2011). This growth has led to an increase in anthropogenic stressors in local coastal ecosystems, with dense coastal development generating polluted storm water runoff and sewage discharge, severely impacting macroalgae and benthic invertebrate cover, fish recruitment, and ecosystem dynamics (Tegner et al. 1995, Bay et al. 2003, Wernberg et al. 2010, Foster and Schiel 2010, Filbee-Dexter et al. 2016), thus potentially compromising the long-term sustainability of our valuable coastal ecosystems.

Maintaining biodiversity is fundamental to the health and resilience of marine ecosystems (Steneck et al. 2002, Folke et al. 2004, Levin and Lubchenco 2008, Bernhardt and Leslie 2013). More biodiverse marine ecosystems display increased connectivity and functional redundancy of species, reducing ecosystem wide responses to local extinction and increasing resilience to both anthropogenic and natural stressors (Peterson et al. 1998, Steneck et al. 2002, Folke et al. 2004, Levin and Lubchenco 2008, Bernhardt and Leslie 2013). In addition, more biodiverse marine ecosystems support greater ecosystem functioning and the continued delivery of crucial

ecosystem services including the food security of over 1 billion people (Daily 1997, Duarte 2000, Worm et al. 2006, Haines-Young and Potschin 2010, Ye et al. 2011, Bolster, Jeff et al. 2012). Therefore maintaining high biodiversity is paramount for promoting global food security and the resilience of marine ecosystems (Worm et al. 2006).

### *Marine Protected Areas as a Conservation Management Tool*

Given the value of marine biodiversity to human society and the severe threats currently facing these ecosystems, marine resource managers are increasingly turning to marine protected areas (MPAs) to increase ecosystem function and resilience of marine ecosystems (Edgar et al. 2007). MPAs can restore biodiversity and abundance of fish species (Murray et al. 1999, Edgar et al. 2007, Lester and Halpern 2008, Claudet et al. 2008, Lester et al. 2009, Ling et al. 2009, Babcock et al. 2010, Hamilton et al. 2010, Thompson et al. 2012, 2017, Harrison et al. 2012, Grorud-Colvert et al. 2014, Wilhelm et al. 2014, Caselle et al. 2015, Starr et al. 2015, Caselle et al. 2018, Hamilton and Caselle 2015, Di Lorenzo et al. 2016, Nickols et al. 2019), have important spillover effects that improve the health of unprotected areas (Edgar et al. 2007, Follesa et al. 2011, Kay et al. 2012, Caselle et al. 2015, Hamilton and Caselle 2015, Di Lorenzo et al. 2016, Fuller et al. 2019), and increase ecosystem resilience to both climate events and overfishing (Edgar et al. 2007, Ling et al. 2009, Babcock et al. 2010, Claisse et al. 2013, Mach et al. 2017, Arafeh-Dalmau et al. 2020). These important benefits have sparked a global increase in MPAs to protect marine fisheries and ecosystems, with over 5.3% of the global oceans now protected in reserves, an increase of 3.3% since I began my Ph.D. (Wood et al. 2008, Lester et al. 2009, Friedlander et al. 2017, Sala and Giakoumi 2018).

California began establishing MPAs around the Northern Channel Islands and on Catalina Island in the 1980s (Airamé et al. 2003, Airame and Ugoretz 2008). In 2003, they

established a new system of reserves to form the Channel Islands National Marine Sanctuary, and expanded these reserves into federal waters in 2006 and 2007 (Airamé et al. 2003, Airame and Ugoretz 2008). In 2012, under the California Marine Life Protection Act, a newly designated network of MPAs was established across the Southern California Bight protecting 16% of state waters (Gleason et al. 2013, Saarman et al. 2013). These MPAs are characterized by different management restrictions including no anchoring zones, limited take of pelagic finfishes and pacific spiny lobster, and no take zones. In addition, the size of the MPAs range widely, from 0.008 to over 40 km<sup>2</sup> (Sprague et al. 2013). Importantly these MPAs cover a broad array of marine ecosystems in the Southern California Bight including sandy bottom, intertidal, and nearshore rocky reef habitats including kelp forest ecosystems (Saarman et al. 2013).

### *Study System*

The Southern California Bight, including the Channel Islands, is a unique marine ecoregion located between the US-Mexican border and Point Conception (32°N and 34.5°N). A distinctive feature of this ecoregion is the oceanographic convergence of the California Current and California Counter Current. This convergence leads to the co-occurrence of both Southern and Northern kelp forest species, making the Southern California Bight a marine biodiversity hotspot (Dailey et al. 1993, Allen and Horn 2006, Block et al. 2011, Stuart-Smith et al. 2013, Schiel and Foster 2015, Piacenza et al. 2015). The subtidal photic habitats of the Southern California Bight biodiversity hotspot are dominated by kelp forest ecosystems, in which the canopy forming *Macrocystis pyrifera* serves as a foundational species for a diverse assemblage of marine fauna (Schiel and Foster 2015). The occurrence of kelp forest ecosystems within the Southern California Bight is highly dependent on the availability of nutrients supplied from seasonal upwelling events. However, the community dynamics of kelp forest fauna also play an



important role in the maintenance and persistence of these biodiverse and highly productive kelp forest ecosystems (Foster and Schiel 1985, Estes and Duggins 1995, Schiel and Foster 2015).

A strong abiotic driver of kelp forest ecosystems within the Southern California Bight are climatic stressors, ranging from the El Niño Southern Oscillation and to climate change-forced marine heat wave events (Dailey et al. 1993, Dayton et al. 1999, Schwing et al. 2003, Gentemann et al. 2017, Rogers-Bennett and Catton 2019). El Niño strongly affects primary productivity, fish population dynamics, and larval dispersal in Southern California Bight ecosystems, leading to large disruptions in ecosystem dynamics including the spatial and temporal reduction in kelp forest biodiversity (Dayton et al. 1999, Wingfield and Storlazzi 2007). El Niño drives elevated sea surface temperatures, lower nutrient concentrations, and increased storm frequency often leading to complete loss of local adult populations of *M. pyrifera*. This loss of *M. pyrifera* then leads to cascading ecosystem effects, notably the loss of *M. pyrifera* associated community members (Dayton et al. 1999, Graham 2004, Wingfield and Storlazzi 2007, Castorani et al. 2018, Rogers-Bennett and Catton 2019). However, despite the dramatic changes of macroalgal communities in response to El Niño events, analysis of kelp forest ecosystems in the Channel Islands suggest that long term perturbations such as sustained overfishing have greater effects on overall ecosystem dynamics (Halpern and Cottenie 2007). Notwithstanding the temporary loss of marine biodiversity, there is strong evidence that Southern California Bight kelp forest ecosystems are resilient to the El Niño and climate stressors, being able to rapidly recover both biodiversity and ecosystem function as was recently demonstrated in the response to the prolonged marine heat wave experienced from 2014-2016 (Dayton and Tegner 1984, Tegner and Dayton 1987, Dayton et al. 1999, Williams et al. 2018, Ruthrof et al. 2018, Lonhart et al. 2019,

Morgan et al. 2019, Rogers-Bennett and Catton 2019, Sanford et al. 2019, Arafeh-Dalmau et al. 2020, Walker et al. 2020, Santora et al. 2020).

In addition to strong natural stressors, the Southern California Bight biodiversity hotspot also experiences severe anthropogenic impacts, drastically altering the region's marine communities (Dailey et al. 1993, Dayton et al. 1998, Foster and Schiel 2010, Schiel and Foster 2015). Historical overfishing in the Southern California Bight has led to large changes in community assemblages with a drastic reduction of top predator species and loss of marine biodiversity (Dayton et al. 1998, Jackson et al. 2001, Foster and Schiel 2010, Hamilton and Caselle 2015, Rossetto et al. 2015, House et al. 2016). In many temperate regions like Southern California, overfishing is strongly associated with trophic cascades leading to the phase shift from kelp forest ecosystems to urchin barren dominated reefs (Behrens 2004, Guidetti 2006, Hamilton and Caselle 2015). Severe localized overharvest of Sea otters (*Enhydra lutris*), California sheephead wrasse (*Semicossyphus pulcher*), and Pacific spiny lobster (*Panulirus interruptus*) in the Southern California Bight led to predatory release of sea urchin species (*Strongylocentrotus franciscanus* and *S. purpuratus*) and subsequent phase shifts from kelp to urchin barren ecosystems (Behrens 2004, Szpak et al. 2012, Hamilton and Caselle 2015).

Fortunately, Southern California kelp forests have demonstrated higher resilience to overfishing pressures compared to other temperate kelp forest ecosystems (Hamilton and Caselle 2015). This increased resilience is thought to be a result of high biodiversity and functional redundancy of urchin predators in Southern California Bight kelp forest ecosystems (Dayton et al. 1998, 1999, Foster and Schiel 2010, Hamilton and Caselle 2015, Braje et al. 2017, Melis et al. 2019, Eisaguirre et al. 2020). However despite higher resilience in these reefs, severe overfishing of large top predators during the 19<sup>th</sup> and 20<sup>th</sup> centuries led to dramatic changes in Southern

California kelp forest dynamics, driving dozens of species to functional extinction (Dayton et al. 1998, Erlandson et al. 2005, Estes et al. 2011, Bolster, Jeff et al. 2012, Chabot et al. 2015, Hamilton and Caselle 2015, Halpern et al. 2015, House et al. 2016, Braje et al. 2017, Thompson et al. 2017, Cabral et al. 2017, Pondella II et al. 2018, Zellmer et al. 2018).

Under future climate change, the Southern California Bight is predicted to face higher sea surface temperatures and ocean acidification with potentially severe consequences for the region's unique marine biodiversity (Dayton and Tegner 1984, Seymour et al. 1989, Dayton et al. 1992, 1999, Ling et al. 2009, Kroeker et al. 2010, 2012, Hofmann et al. 2011, Doney et al. 2012, Muller-Karger et al. 2018, Ruthrof et al. 2018, Rogers-Bennett and Catton 2019, Sanford et al. 2019, Bednaršek et al. 2020). The severe consequences of climate change driven marine heat waves have already been observed in the functional extinction of *Pycnopodia helianthoides* along much of the California coast and the near total deforestation of Northern California kelp forest habitats (Elder et al. 2016, Schultz et al. 2016, Moritsch and Raimondi 2018, Winningham et al. 2018, Ruthrof et al. 2018, Harvell et al. 2019, Morgan et al. 2019, Rogers-Bennett and Catton 2019, Arafeh-Dalmau et al. 2020). In kelp forest ecosystems, increased SST also is linked with decreased abundance of native canopy forming macroalgae including *M. pyrifera*, and an increase of warmer water species (Dayton et al. 1999, Schiel et al. 2004, Halpern et al. 2008, Vergés et al. 2016, Williams et al. 2018, Morgan et al. 2019, Rogers-Bennett and Catton 2019, Sanford et al. 2019, Arafeh-Dalmau et al. 2020). Local anthropogenic thermal stress has been demonstrated to cause dramatic shifts in the native macroalgae species and subsequent loss of macroinvertebrates and fish associated communities (Schiel et al. 2004, Ruthrof et al. 2018, Harvell et al. 2019, Morgan et al. 2019, Rogers-Bennett and Catton 2019, Arafeh-Dalmau et al. 2020). In addition there is already substantial evidence that both Southern California Bight and

global temperate fish assemblages have shifted toward warm water species as a response to climate driven SST increases over the past century (Holbrook et al. 1997, Graves et al. 2006, Last et al. 2011, Vergés et al. 2016, Williams et al. 2018, Sanford et al. 2019, Walker et al. 2020). Together these studies suggest that climate change driven increases in SST will continue to drive ecosystem communities towards neotropical assemblages and lead to the loss of important foundational canopy forming macroalgal species (Dayton et al. 1999, Schiel et al. 2004, Doney et al. 2012, Przeslawski et al. 2012, Rogers-Bennett and Catton 2019, Arafah-Dalmau et al. 2020). Furthermore, climate change has the potential to greatly alter the frequency and intensity of El Niño events, with potentially synergistic impacts on marine biodiversity and ecosystem functioning within the Southern California Bight (Halpern and Cottenie 2007, Wingfield and Storlazzi 2007).

Compounding increased temperatures is increased ocean acidification within the Southern California Bight over the past century (Fabry et al. 2008, Doney et al. 2009, Kroeker et al. 2010, Bednaršek et al. 2020). Increased ocean acidification strongly reduces the growth and increase the mortality of marine calcifying organisms within kelp forest ecosystems, potentially leading to the loss of key benthic invertebrates (Wootton et al. 2008, Kroeker et al. 2010, 2012, Hofmann et al. 2011, Melzner et al. 2012, Bednaršek et al. 2020). In addition, differential evolutionary potential to adapt to ocean acidification could favor the success of functionally important taxa like the red sea urchin (*Strongylocentrotus franciscanus*) (Sunday et al. 2011), with potential negative impacts on kelp forests. Furthermore, decreased pH can favor non-calcifying and turf macroalgae over foundational canopy forming species (Kroeker et al. 2012) and impair sensory capabilities, potentially disrupting ecologically important predation and migration patterns of kelp forest fish species (Munday et al. 2009b, 2009a, Dixon et al. 2010,

Cripps et al. 2011, Simpson et al. 2011), processes that could catalyze drastic changes in marine biodiversity across the Southern California Bight.

Furthermore, intensive urbanization of the Southern California Bight's coastal environments has drastically altered marine ecosystems, leading to severe losses of marine biodiversity and erosion of ecosystem health (Shahidul Islam and Tanaka 2004, Halpern et al. 2008, Diaz and Rosenberg 2008). The impacts of urbanization are particularly concerning in the Los Angeles metropolitan area which has developed into one of the world's largest urban environments, drastically altering hydraulic, nutrient, and biogeochemical processes over the past century (Ahn et al. 2005, Alberti 2005, Feagin et al. 2005, Sheng and Wilson 2009, Pataki et al. 2011). This growth has led to an increase in anthropogenic stressors in local coastal ecosystems, with dense coastal development generating polluted storm water runoff and sewage discharge, severely impacting kelp cover, fish recruitment, and ecosystem dynamics (Tegner et al. 1995, Duke et al. 1998, Haile et al. 1999, Bay et al. 2003, Boehm Jr et al. 2009, Wernberg et al. 2010, Foster and Schiel 2010, Gold et al. 2013, Filbee-Dexter et al. 2016, Gallo et al. 2020). Furthermore, severe nutrient pollution associated with sewage discharges can result in decreased abundance of native macroalgae, resulting in shifts to less diverse urchin barren ecosystems (Dayton et al. 1992, Tegner et al. 1995, Foster and Schiel 2010).

Furthermore, Los Angeles is home to the largest port in the United States and fourth largest in the world, with an enormous industrialization foot print and daily movement of hundreds of massive transoceanic vessels bringing ballast water and potential invasive species (Ahn et al. 2005, Lemus 2005). Numerous invasive species, including the highly invasive brown algae *Sargassum horneri* or "Devil Weed", have been introduced to the Southern California Bight through the port of Los Angeles and dumping of home aquaria (Marks et al. 2015). These

species often have complex effects to on ecosystems, such as the recent *S. horneri* invasion which has led to the short term loss and displacement of native *Macrocystis pyrifera* in many rocky reefs around Southern California (Tanner n.d., Marks et al. 2015, Caselle et al. 2018). Together historical and continued urbanization of the Southern California Bight potentially compromises the long-term sustainability of the regions valuable coastal and marine ecosystems.

### *Monitoring Marine Protected Areas*

In order to address the effects of these anthropogenic impacts, marine managers have established a network of marine protected areas (MPAs) within the Southern California Bight region (Gleason et al. 2013, Saarman et al. 2013). Since the creation of these MPAs, there has been evidence that these reserves have helped successfully restore marine ecosystems, specifically in response to reduce fishing pressure (Airame and Ugoretz 2008, Hamilton et al. 2010, Pondella II et al. 2015, 2019, Caselle et al. 2015, Starr et al. 2015, Cabral et al. 2017). In the Channel Islands, the abundance of targeted fish species has increased significantly faster within MPAs compared to outside the reserves (Airame and Ugoretz 2008, Hamilton et al. 2010, Caselle et al. 2015). Furthermore, MPAs have seen a significant decline in the number of urchin barrens within Channel Islands MPAs, likely driven by the increased abundance of both *S. pulcher* and *P. interruptus* species (Airame and Ugoretz 2008, Kay et al. 2012, Hamilton and Caselle 2015). Thus, there is evidence that the MPAs established within the Channel Islands have been effective in restoring marine biodiversity and ecosystem health (Pondella II et al. 2015).

However, these previous evaluations of the MPA network have taken place with relatively limited data. Evaluating the effectiveness of MPAs requires reliable ways to survey and evaluate marine biodiversity and ecosystem function. Important data for evaluating

ecosystem function within marine ecosystems include fish biodiversity, abundance, and trophic structure assessment (Edgar et al. 2007, Usseglio 2015). Current methods for observing nearshore marine ecosystems rely heavily on visual SCUBA-based surveys (Hodgson et al. 2004, Sprague et al. 2013) and are thus effected by observer biases (Edgar et al. 2004). In the case of visual fish census methods, observer bias can result from a variety of factors including species response to the presence of divers (Lindfield et al. 2014), taxonomic expertise (Edgar et al. 2004), dive conditions (Bozec et al. 2011), and mismatch between species activity and time surveys are conducted (Bassett and Montgomery 2011). In particular, visual fish surveys often fail to capture transient and migratory predators that rarely utilize one particular reef, but can play an outsize role in ecosystem structuring (Lowe et al. 2006, Thomsen et al. 2016, Bakker et al. 2017, Lafferty et al. 2018). Also difficult to monitor are cryptic benthic species that require careful and time consuming observation in and around crevices and macroalgae (Willis 2001).

In addition to the above, worker safety constraints limit the length and depths of dive surveys, typically to 1-2 hours duration and less than 30m in depth (Edgar et al. 2004). Even with such a limited scope, visual scuba surveys are still expensive, often requiring large and specialized vessels and crews of trained divers (Hodgson et al. 2004, Sprague et al. 2013). Combined, these issues largely limit monitoring efforts to economically important and conspicuous fish species, while restricting surveying efforts to only a handful of sites, typically once per year as is the case with monitoring Southern California MPAs (Willis 2001, Costa et al. 2013, Grorud-Colvert et al. 2014, Pondella II et al. 2015, Caselle et al. 2015). This limited data makes it difficult to accurately assess the health of this MPA network, especially in response to global change. As such, new approaches to monitor MPA health – ones that produce data on

broader taxonomic, spatial and temporal scales – will be required to evaluate effectiveness of the Southern California MPA Network (Gleason et al. 2013, Saarman et al. 2013).

### *Environmental DNA*

One increasingly popular approach to biodiversity monitoring is environmental DNA (eDNA) metabarcoding which offers a rapid, cost effective, and accurate tools for monitoring fish biodiversity (Taberlet et al. 2012a, Kelly et al. 2014a, Miya et al. 2015). eDNA metabarcoding relies on the capture and sequencing of DNA left behind by resident marine species, allowing for the reconstruction of communities from the collection and processing of sea water (Ficetola et al. 2008).

Advances in eDNA techniques have allowed scientists to accurately detect over 93% of fish species from seawater in both aquaria and reef ecosystems (Thomsen et al. 2012, Kelly et al. 2014a, Port et al. 2015, Miya et al. 2015, Valentini et al. 2016). However, previous comparisons of eDNA and visual methods reveal overlapping but different views of marine communities (Thomsen et al. 2012, 2016, Kelly et al. 2014a, 2017, Ushio et al. 2018a, Closek et al. 2019). Almost universally, eDNA detects a broader range of taxa than visual surveys (Port et al. 2015, Valentini et al. 2016, Yamamoto et al. 2016, Ushio et al. 2018b), but frequently fails to detect a small subset of species observed visually, resulting in differing community patterns across sites (Kelly et al. 2017). Furthermore, species abundance, not just diversity, is a critical measures of marine ecosystem health (Tegner 2000, Edgar et al. 2007, Lester and Halpern 2008, Gaines et al. 2010), and the utility of eDNA to estimate abundance remains equivocal (Iversen et al. 2015, Evans et al. 2016, Klobucar et al. 2017, Fonseca 2018, Rice et al. 2018, Chambert et al. 2018, Tillotson et al. 2018, Pont et al. 2018). Thus, despite its promise, eDNA research is still in its infancy.



In particular, eDNA metabarcoding approaches are hampered by bioinformatic challenges to accurately assign species level identification including both a lack of available reference barcode sequences as well as flexible, accurate, and fast pipelines to process and analyze multi-locus metabarcoding data (Curd et al. 2019). Furthermore, few studies have directly compared eDNA and visual estimates in marine ecosystems to test whether they yield similar ecological patterns (Kelly et al. 2014b, Bohmann et al. 2014), information that is critical to inform future applications of eDNA metabarcoding approaches for monitoring MPAs.

Thus my thesis aims to addressing these above issues through 1) the generation of accurate and comprehensive reference database for California coastal marine fishes, 2) assessment of eDNA and underwater visual survey methods inside and outside a no-take reserve, and 3) comparison of eDNA metabarcoding and underwater visual survey methods broadly across the Southern California MPA network. In addition, I include additional efforts on the development of an eDNA bioinformatic pipeline for processing multi-locus metabarcoding data as well as the application of eDNA metabarcoding approaches to surveying endangered fairy shrimp species in vernal pool habitats.

## CHAPTER 1

### **FishCARD: Fish *12S* California Current Specific Reference Database for Enhanced Metabarcoding Effort**

#### **Abstract**

DNA metabarcoding is an important tool for molecular ecology. However, metabarcoding effectiveness hinges on the quality of reference databases for taxa and loci of interest. This limitation is true for metabarcoding of marine fishes in the California Current Large Marine Ecosystem where there is a paucity of reference *12S* barcodes. Here we present FishCARD, a California Current-specific fish *12S*-specific reference barcode database. We barcoded 612 species using the MiFish metabarcoding primers; an addition of 258 species to the 459 California Current fish species with existing *12S* barcodes from GenBank. The resulting FishCARD database covers 82.7% of California Current fishes, and it includes virtually all fishes sampled by large marine monitoring programs such as the Partnership for Interdisciplinary Studies of Coastal Oceans and California Cooperative Oceanic Fisheries Investigation. To demonstrate the importance of complete reference databases for eDNA metabarcoding, we compared species and reads identified from three 1L seawater samples collected off Santa Cruz Island, CA using GenBank sequences with and without our generated barcodes, as well as the FishCARD database curated here. The inclusion of our generated barcodes allowed the additional identification of 15 native taxa and 21.8% of total reads from eDNA samples. However, we found that half of all amplicon sequence variants (ASVs) generated by MiFish *12S* primers were of non-vertebrate *16S* origin, demonstrating a clear limitation of a widely employed

fish metabarcoding primers. Despite these limitations, FishCARD provides an important genetic resource to enhance the effectiveness of marine metabarcoding efforts in the California Current Large Marine Ecosystem.

## **Introduction**

Next generation DNA sequencing provides advanced tools for marine ecology and ecosystem monitoring (Kelly et al. 2014b, Closek et al. 2019, Yamahara et al. 2019). The ability to sequence tens to hundreds of millions of reads in a single sequencing run allows for the development of novel genomic applications to a suite of research questions including species mapping, biomonitoring, gut content analyses, and population genomics, all of which aid our understanding of the ecology of marine ecosystems (Sanders et al. 2015, Guo 2017, Thompson et al. 2017, Baetscher et al. 2019).

Key to these advances is next-generation sequencing metabarcoding. Metabarcoding is a process in which multiple species are identified from bulk DNA (e.g., homogenized gut contents or settlement tile scrapings) or environmental DNA (eDNA) samples (e.g., water and soil) typically by PCR amplification and sequencing of a target gene, and then comparing the resulting DNA sequences to a database of known reference sequences (Taberlet et al. 2012a). In particular, the application of eDNA metabarcoding allows researchers to detect a broad range of marine diversity from a single liter of seawater and has the potential to dramatically improve marine biomonitoring efforts (Kelly et al. 2014b).

The success of metabarcoding approaches relies on the quality of reference databases, specifically their completeness and accuracy (Boyer et al. 2016, Machida et al. 2017). The absence of reference barcodes for a given species for a target locus makes it impossible to accurately classify all sequences generated through metabarcoding with current bioinformatic

technology (Deiner et al. 2017). Inadequate reference databases are an acute problem for barcoding, metabarcoding, and eDNA studies that limit the accuracy of taxonomic identification and have the potential to bias the interpretation of results (Andruszkiewicz et al. 2017b, Klymus et al. 2017, Djurhuus et al. 2020). Thus building complete and accurate reference databases is paramount to the success of molecular ecology monitoring efforts (Schenekar et al. 2020). To address the need for accurate and complete reference databases, previous efforts were made to barcode California Current Large Marine Ecosystem fishes focused on the mitochondrial Cytochrome Oxidase I (*COI*) locus (Hastings and Burton 2008, Ward et al. 2009, Ardura et al. 2013, Duke and Burton 2020).

However, recent metabarcoding studies of marine fishes have focused instead on a short segment of the mitochondrial *12S* RNA gene because it provides species-level resolution for many fishes while being vertebrate-specific (Miya et al. 2015, Valsecchi et al. 2019). The smaller *12S* locus is also thought to be advantageous for eDNA studies because of DNA isolated from the environment tends to be degraded and commonly used sequencing technologies target relatively small loci (Miya et al. 2015, Jo et al. 2017, Collins et al. 2019). Given the success of this metabarcoding primer set, the MiFish Universal Teleost primer set is the most commonly used *12S* barcode region because of its utility across a diverse assemblage of marine fishes (Thomsen et al. 2016, Bista et al. 2017, Yamamoto et al. 2017, Closek et al. 2019, Valsecchi et al. 2019).

Thus while there is a near complete *COI* barcode database of California Current fishes (Hastings & Burton, 2008), there is a relative lack of *12S* barcodes for California Current fishes in existing reference databases; GenBank has MiFish *12S* barcodes for 459 of the 864 California fish species (NCBI download October 2019). This paucity of barcodes severely limits the utility

of *12S* metabarcoding approaches in California Current coastal waters (Port et al. 2015, Andruszkiewicz et al. 2017b, Djurhuus et al. 2020), where relatively recently established marine protected areas (Thompson et al. 2012, Gleason et al. 2013, Pondella II et al. 2015) have created an urgent need for effective and economical monitoring (Harada et al. 2015, Duke et al. 2018).

Metabarcoding has the ability to help marine resource managers address critical questions, ranging from shifting species distributions, effectiveness of marine protected areas, and seasonal patterns of larval fish recruitment, among others (Kelly et al. 2014b, Closek et al. 2019, Djurhuus et al. 2020, Duke and Burton 2020). However, the success of metabarcoding efforts to enhance fishery management in the California Current Large Marine Ecosystem depends on the development of an improved *12S* barcode reference database. Towards this end, we developed the FishCARD reference database. This regionally-specific database is curated for marine fishes found in the California Current Large Marine Ecosystem, comprised of *12S* sequences previously available in GenBank supplemented by hundreds of additional *12S* sequences generated during this study.

## **Methods**

### *Reference Barcode Generation*

To generate a more complete *12S* barcode reference database for fish found in the California Current Large Marine Ecosystem, we assembled a list of native marine teleost and elasmobranchs, comprising a total of 864 species (Allen & Horn, 2006; Froese & Pauly, 2010; Hastings & Burton, 2008; Love, & Passarelli, 2020) (Supplemental Table 1-S1). From this list, we acquired as many ethanol-preserved specimens as possible from the Scripps Institution of Oceanography Marine Vertebrates Collection at University of California San Diego (SIO). We

obtained a total of 757 samples, representing 612 species (Supplemental Table 1-S2) or 70.8% of all described species of California Current marine fishes. Of these 757 samples, 258 had no previous *I2S* barcodes.

For each sample, we extracted DNA from ~0.25 mg of tissue in 300  $\mu$ L of a 10% Chelex slurry (Walsh et al. 1991). Given the high volume of samples to process, we initially froze sample slurries at -20°C. Subsequently, samples were thawed, vortexed for 10 seconds, and then centrifuged at high speed for 15 seconds prior to incubating at 95°C for 20 minutes. Samples were then vortexed and centrifuged again at high speed and stored at 4°C until use.

We amplified all DNA extracts using the MiFish Universal Teleost Primers and additionally amplified all elasmobranch samples using the MiFish Elasmobranch Primers (Miya et al. 2015). PCR amplification was conducted following the thermocycler profile of Curd et al. (2019). PCR reactions had 25  $\mu$ L reaction volume containing 12.5  $\mu$ L QIAGEN Multiplex Taq PCR 2x Master Mix (Qiagen Inc., Valencia, CA, USA), 6.5  $\mu$ L of molecular grade water, 2.5  $\mu$ L of each primer (2  $\mu$ mol/L), and 1  $\mu$ L DNA extraction. PCR thermocycling employed a touchdown profile with an initial denaturation at 95°C for 15 min to activate the DNA polymerase, followed by 13 cycles of a 30s denaturation at 94°C, a 30s annealing that started at 69.5°C and then decreased by 1.5°C for each subsequent cycle (last cycle was 50°C), finishing with a 1 min extension at 72°C. This initial touchdown profile was followed by 35 additional cycles using identical parameters except a constant annealing temperature of 50°C and ending with a final extension at 72°C for 10 min. All PCRs included a negative control, where molecular grade water replaced the DNA extraction. All PCR products were visualized via electrophoresis on 2% agarose gels to ensure amplification success and correct product size.

PCR products were purified using ExoSAP-IT (Affymetrix, Cleveland, OH, USA) and sequenced in both directions using BigDye chemistry (Applied Biosystems Inc, Foster City, CA, USA) at Laragen Inc., (Culver City, CA, USA). We trimmed and aligned forward and reverse sequences in Sequencher version 5.4.6 (Nishimura 2000). All taxonomic names between GenBank and voucher specimens were synonymized to NCBI taxonomy using the *R* package *taxize* (Chamberlain and Szöcs 2013). The resulting *12S* sequences will be deposited into GenBank upon publication (Supplemental Table 1-S2).

### *eDNA Metabarcoding*

To test the utility of the FishCARD database, we metabarcoded fish eDNA from 3 sites off Eastern Santa Cruz Island, CA in 2017. We collected seawater samples from 10m depth using a 4 L Niskin bottle. From this sample, we gravity filtered 1 L through a 0.2  $\mu$ m Sterivex (Millipore Sigma, Burlington, MA, USA) filter in the field and preserved on ice before being transported to a -20°C freezer. We then extracted eDNA from the filters using the modified Qiagen DNeasy Blood and Tissue Kit extraction protocol of Spens et al. (2017), and then amplified it via PCR using the MiFish Teleost primers and thermocycler profile above. We prepared libraries following the methods of Curd et al. (2019) and sequenced these samples on an Illumina MiSeq PE 2x300 at UCLA Technology Center for Genomics and Bioinformatics (see Appendix A – Supplemental Methods for detailed library preparation protocol).

### *Bioinformatics and Taxonomic Assignment*

We processed DNA sequences using the *Anacapa Toolkit* (Curd et al. 2019) following default parameters with a Bayesian cutoff score of 60. We then assigned taxonomy to each generated amplicon sequence variant (ASV), unique sequence generated through metabarcoding,

using three different reference databases. First, we created a *12S* reference database using *CRUX* (Curd et al. 2019), which compiled all publicly available matching *12S* barcode sequences from the NCBI GenBank database targeted by the MiFish Universal Teleost primers, employing standard *CRUX* parameters (Benson et al., 2018; Curd et al., 2019). This set of sequences is herein referred to as the “*CRUX-12S* database” and included any GenBank reference barcode that *in silico* amplified to the MiFish *12S* primers (sequences downloaded in October 2019; <https://github.com/zjgold/FishCARD>). Second, to evaluate how increasing database coverage improves taxonomic assignments, we supplemented the *CRUX-12S* database with the 757 additional California Current fish *12S* barcodes generated for this study, herein referred to as the “combined database”. Third, to test the value of a database curated for the region, we created a reference database comprised of only *12S* barcodes of fishes native to California Current Large Marine Ecosystem. These sequences included those obtained from GenBank via *CRUX* and the 757 newly generated reference sequences. This regionally specific reference database is subsequently referred to as “FishCARD”.

### *Reference Database Comparisons*

To compare the effectiveness of the three reference databases, we examined the total number of ASVs and taxonomic ranks identified using each database. We also investigated differences in taxonomic assignment between single direction ASVs (comprised of forward- and reverse-only sequence reads) and merged ASVs (merged paired-end sequence reads). Specifically, we compared the *CRUX-12S* database to the combined database to determine how the inclusion of additional region-specific reference barcodes improved taxonomic assignment of California Current fish species from eDNA samples. Next, we compared taxonomic assignments between the FishCARD California Current-specific *12S* reference database and the combined



reference database to determine whether a curated metabarcoding reference database specific to a regional fauna performs better than a database that uses all available *12S* reference barcodes.

## Results

### *Reference Barcode Generation*

We generated 757 *12S* MiFish barcodes for 612 California Current fishes, 557 teleosts, 51 elasmobranchs, and 4 cyclostomatan (Supplemental Table 1-S2). Of these, 56 barcodes were duplicates of the same elasmobranch taxa amplified with both the MiFish Elasmobranch and MiFish Universal *12S* primer sets. In total, we generated an additional 258 novel *12S* barcodes for California Current fishes compared to what was available in GenBank at the time of publication. Combining these barcodes with existing reference sequences deposited on GenBank (October 2019) provides reference *12S* barcodes for 715 out of 864 (82.8%) California Current fish species.

### *eDNA Metabarcoding*

We generated a combined 330,877 sequence reads from 3 eDNA samples, resulting in a total of 2,152 ASVs including singletons; this total dropped to 341 ASVs excluding singletons. Of these 341 ASVs, 211 ASVs were merged paired-reads, 123 ASVs were forward-only reads, and 7 ASVs were reverse only reads. All comparisons below excluded singletons (ASVs that occurred only once across the three samples).

### *Unassigned MiFish 12S ASVs*

The *Anacapa Toolkit* failed to assign taxonomy to 49.6% (169/341) of ASVs representing 24.5% (81,002/330,877) of all reads using all three reference databases investigated in this study

(Supplemental Table 1-S4). Of the 169 unassigned ASVs, 16 were forward-only reads, and 153 were merged reads. To explore the origins of these unassigned reads, we used BLAST to query all GenBank sequences, revealing that 94.7% (160/169) of these ASVs aligned to marine prokaryotic and eukaryotic *16S* sequences (Max Alignment Scores 87.9-475). Of these aligned ASVs, 85% (136/160) matched to uncultured sequences generated from marine metagenomic studies. 80.0% (128/160) of successfully aligned ASVs matched to bacterial barcodes including those from *Psychromonas* sp., *Photococcus caeruleum*, *Loktanella* sp., *Leucothrix* sp., and *Gimesia* sp., and cyanobacteria. A smaller fraction of assigned ASVs (18.8%; 30/160) best aligned to eukaryotic sequences including those from diatoms (e.g. *Nitzschia alba* and *Eucampia antarctica*) and other marine microalgae (e.g. Picobiliphytes, *Heterosigma akashiwo*, *Mesopedinella arctica*, and *Phacus warszewiczii*). Given that these 169 unassigned sequences were non-vertebrate, we excluded these ASVs from all subsequent comparisons. All remaining 172 ASVs were assigned to a Class of vertebrates by at least one of the three reference databases used. Of these vertebrate ASVs, 58 were merged, 107 were forward-only, and 7 were reverse only reads.

#### *Reference Database Comparisons*

Samples processed using the *CRUX-12S* reference database assigned 89.5% (154/172) of vertebrate ASVs to Family-level and 84.3% (145/172) of vertebrate ASVs to species-level (Supplemental Table 1-S5). Examining merged reads only, the *CRUX-12S* reference database assigned 94.8% (55/58) of all vertebrate ASVs to Family-level and 87.9% (51/58) of all vertebrate ASVs to species-level (Supplemental Table 1-S6). Examining only forward-reads, the *CRUX-12S* database assigned 87.9% (94/107) of all vertebrate ASVs to Family-level and 83.2% (89/107) of all vertebrate ASVs to species-level. Across all vertebrate ASVs, the *CRUX-12S*

database produced 31 unique Family-level assignments, 39 unique genus-level assignments, and 38 unique species-level assignments of which only 25 were species native to the California Current Large Marine Ecosystem. Across all vertebrate ASVs, the *CRUX-12S* database failed to resolve 1.7% (3/172) of vertebrate ASVs to a vertebrate Class, 7% (12/172) of vertebrate ASVs below Class-level, and 1.7% (3/172) of vertebrate ASVs below Order-level.

We then conducted taxonomic assignments using the combined database comprised of both *CRUX-12S* reference barcodes supplemented with the additional 757 barcodes generated in this study. Samples processed using the combined reference database assigned 100% (172/172) of vertebrate ASVs to Family-level and 88.4% (152/172) of vertebrate ASVs to species-level. For merged reads only, the combined database assigned 100% (58/58) of all vertebrate ASVs to Family-level and 93.1% (54/58) of all vertebrate ASVs to species-level. For forward-only reads, the combined database assigned 100% (107/107) of all vertebrate ASVs to Family-level and 87.9% (94/107) of all vertebrate ASVs to species-level. Across all vertebrate ASVs, the combined database produced 28 unique Family-level assignments, 38 unique genus-level assignments, and 37 unique species-level assignments of which 36 were species native to the California Current Large Marine Ecosystem. No ASVs were assigned to only the Class- or Order-level.

Lastly, we assigned taxonomy using the curated FishCARD reference database comprised of only California Current fish *12S* reference barcodes. Samples processed using the curated FishCARD reference database resulted in 99.4% (171/172) of all ASVs assigned to Family-level and 90.7% (156/172) of all ASVs assigned to species-level. For merged reads only, the FishCARD database assigned 100% (58/58) of all vertebrate ASVs to Family-level and 95% (55/58) of all vertebrate ASVs to species-level. For forward-only reads, the FishCARD database

assigned 100% (107/107) of all vertebrate ASVs to Family-level and 86.0% (92/107) of all vertebrate ASVs to species-level.

Across all vertebrate ASVs, the FishCARD database produced 27 Family-level assignments, 39 genus-level assignments, and 37 species-level assignments of which all were native CA species. The FishCARD database failed to assign one vertebrate ASV which was assigned to the Family Delphinidae by both the *CRUX-12S* and combined databases.

### *Reference Database Comparisons*

#### *CRUX-12S* Database vs. Combined Database

Comparing the *CRUX-12S* and combined database results demonstrated that the inclusion of novel voucher sequences allowed for species-level identification for 11 additional California Current taxa including Kelp bass (*Paralabrax clathratus*), California moray (*Gymnothorax mordax*), Opaleye (*Girella nigricans*), Giant kelpfish (*Heterostichus rostratus*), Ocean whitefish (*Caulolatilus princeps*), and California halibut (*Paralichthys californicus*) (Supplemental Table 1-S3). The use of the FishCARD reference database also resulted in improved taxonomic classification for 4 species previously missing barcodes that were assigned to other native California Current fish species using the combined database (Supplemental Table 1-S3).

Surprisingly, the inclusion of additional California Current-specific fish *12S* barcodes also led to 10 ASVs receiving a less specific taxonomic classification compared to the *CRUX-12S* database alone. Ten ASVs assigned to the California native Señorita (*Oxyjulis californica*) by the *CRUX-12S* database were only identified to the Family Labridae using FishCARD.

#### Combined Database vs. Curated Database

The curated California-specific FishCARD database and the combined *CRUX-12S* reference database supplemented with the additional California-specific *12S* barcodes differed for three taxonomic assignments. First, the curated FishCARD database assigned an ASV to the Black croaker (*Cheilotrema saturnum*) that was previously only assigned to the family Sciaenidae by the combined database. Second, the curated FishCARD database identified one ASV as native Bat ray (*Myliobatis californica*) whereas the combined database assigned this ASV to the non-native common Eagle ray (*Myliobatis aquila*). Third, FishCARD reference database failed to resolve one ASV previously assigned to the family of Delphinidae by the combined database.

## **Discussion**

Whether used alone or in combination with existing reference databases, FishCARD reference barcodes dramatically improve the accuracy of eDNA metabarcoding assignments from California Current coastal waters, including species for recreational and commercial fishing and marine ecosystem assessments (Allen and Horn 2006, Sprague et al. 2013, Pondella II et al. 2015). In a test eDNA dataset from 3 sites on Santa Cruz Island, FishCARD performed better, identifying ASVs to species for an additional 15 California Current fishes that were not identified by the *CRUX-12S* database. This increase in accuracy greatly improves the utility of eDNA for monitoring California Current coastal ecosystems, echoing previous research on the importance of complete reference databases in metabarcoding (Leray et al. 2012, Machida et al. 2017).

Unexpectedly, almost half of the ASVs and a quarter of all sequences generated in our eDNA test datasets could not be assigned to species. While other metabarcoding studies report similar results (Leray and Knowlton 2017) (Supplemental Table 1-S1), further investigation

showed that the vast majority of unassigned ASVs were not fish. Instead, they mapped to uncultured bacteria *16S* loci derived from marine shotgun sequencing metagenomic studies (Bork et al. 2015). This unexpected result highlights that the MiFish Teleost *12S* primer set, while extremely useful for targeting vertebrate *12S* loci, can also amplify non-target *16S* genes, potentially inflating the number of ASVs unassigned to species using this primer set.

### *Importance of Complete Reference Databases*

Previous eDNA metabarcoding efforts in the California Current reported poor species-level identification and frequent taxonomic assignment to non-native sister taxa (Kelly et al. 2014a, Port et al. 2015, Closek et al. 2019), results that are likely due to the lack of adequate reference sequences for these species. For example, an eDNA metabarcoding study in Southern California (Curd et al. 2019) assigned multiple *12S* ASVs to *Girella simplicidens*, the Gulf opaleye, a fish native to the Gulf of California that does not occur in California coastal waters (Froese and Pauly 2010, Love and Passarelli 2020). This incorrect assignment occurred because there were no corresponding *12S* reference sequences for the local native Opaleye, *G. nigricans*. By maximizing the number of reference barcodes from local species, FishCARD allows the vast majority of reads to be correctly assigned to ecologically and geographically relevant species.

Compared to the *CRUX* database generated from *12S* fish sequences in *CRUX-12S*, FishCARD improved species-level assignments, identifying an additional 21.8% of the vertebrate reads in our eDNA samples. Much of this improvement was due to the inclusion of reference barcodes for Kelp bass (*Paralabrax clathratus*), one of the most abundant marine species in Southern California kelp forest ecosystems and an important sport fishery species (Pondella II et al. 2015). By including this species, FishCARD assigned 2 previously unidentified ASVs to *P. clathratus*, which accounted for 16.4% of our total sequence reads.

### *Importance of regional reference databases*

Given that increasing reference database completeness increased the ability to assign ASV's to species, it is logical to assume that databases with more taxonomic coverage are better. However, our results suggest an unexpected trade-off between greater diversity of barcodes and regionally/ecologically informed taxonomic assignment. For example, using only the FishCARD database, which is specific to California Current marine fishes, we identified important native taxa like Black croaker (*Cheilotrema saturnum*) and Bat ray (*Myliobatis californica*) in eDNA samples. However, when FishCARD and the *CRUX-12S* databases were combined to yield a database with the largest total number of barcodes, black croaker was not identified and bat ray inconsistently identified across multiple ASVs. The combined database failed to identify black croaker due to the high similarity of *12S* barcode sequences within the Family Sciaenidae, specifically within the clade that includes *Cheilotrema*, a genus native to California, as well as *Equetus* and *Pareques*, non-native coral reef associated genera; Supplemental Table 1-S3). Similarity of barcode sequences also explains the loss of taxonomic resolution in *Myliobatis*.

By excluding highly similar non-native barcodes, the curated FishCARD database provided more accurate species-level assignments, suggesting that a database comprised of only local taxa is preferred to maximize identification of local species. However, this improvement was not universal. For example, FishCARD failed to classify an ASV belonging to the family Delphinidae that was identified by both the CRUX and combined databases. This result stems from FishCARD being specific to California Current fishes and does not include marine mammals. This shortcoming could be easily overcome, however, by appending FishCARD with barcodes for other marine-associated vertebrate taxa of local management interests (Valsecchi et al. 2019).

These results highlight the tradeoff between identifying local species from clades with little genetic variation and providing taxonomic coverage across a broad range of vertebrate species. As such, researchers need to identify their research priorities when deciding on which reference databases to use, with a particular focus on defining the scope of the target taxa. Future work could alleviate this tradeoff by building bioinformatic pipelines that prioritize assignments to a reference set of native species, perhaps by including information on species ranges and sample locations in the assignment algorithm. Alternatively, a regional database could be appended to address specific questions, such as testing for the presence of specific invasive species or range shifts associated with climate change.

#### *Importance of full-length amplicons*

Although FishCARD typically increased taxonomic resolution of ASVs from our eDNA samples, in one specific case the inclusion of additional California Current-specific *12S* barcodes led to substantially reduced taxonomic resolution, with 10 ASVs initially assigned to *Oxyjulis californica* subsequently assigned only to family, Labridae. This result appears to suggest that inclusion of barcodes from local fauna decreases assignment accuracy. However, all 10 of these ASVs were forward sequences only. The Señorita (*Oxyjulis californica*) and Rock wrasse (*Halichoeres semicinctus*), both native to the California Current share a high degree of similarity in the forward *12S* sequence fragment, and the diagnostic SNPs occur in the reverse sequence fragment. This issue is resolved when using the full-length merged reads which capture diagnostic SNPs between these two species. Given genetic similarities between these two species, a recent phylogenetic study of new world *Halichoeres* wrasses has even argued for synonymizing *Oxyjulis* into *Halichoeres* (Wainwright et al. 2018).



Despite the above, single direction reads can provide accurate taxonomic assignment for many California Current fishes. For example, within Labridae, the same family as Señorita and Rock wrasse, forward-reads provided species-level resolution for the California sheephead, *Semicossyphus pulcher*. Such variation in taxonomic assignment based on single-direction sequences highlights the importance of full-length merged reads. To ensure the most accurate taxonomic classifications, researchers should maximize the generation of merged reads by choosing the appropriate sequencing platform (e.g. paired end 300 bp vs. paired end 75 bp).

Although we conducted 300 bp paired-end high throughput sequencing for a 176 bp amplicon, we still obtained a substantial number of orphaned single end reads. This result is largely due to the generation of low quality reverse sequence reads, a persistent issue found across Illumina sequencing platforms (Kwon et al. 2013, Callahan et al. 2016). As such, it is important to think carefully about whether or not to include single end reads on a case-by-case and potentially species-by-species basis. In the present study, we were only able to make this determination because of the relative completeness of the FishCARD database, which included all labrids native to the California Current.

### *Limitations of FishCARD*

The FishCARD database did not include barcodes for all California Current marine fishes due to a combination of limited resources, difficulties amplifying vouchered tissue samples, and a lack of some vouchered reference material within the Marine Vertebrates Collection of the Scripps Institution of Oceanography. Of the 149 (17.2%) California Current fishes absent from FishCARD, 44.0% (n=66) are rare in the California Current, 14.7% (n=22) are common but not coastal species, and 2.0% (n=3) were introduced estuarine species; only, 18.0% (n=27) were common coastal species (Supplemental Table 1-S7). As such, FishCARD provides coverage for

the vast majority of California Current marine fishes, making it an important tool for metabarcoding studies, despite these missing taxa.

The one major shortcoming of FishCARD is that 20.8% (n=31) of the missing taxa are in the Genus *Sebastes*, and rockfish are ecologically important (Hyde and Vetter 2007), form the basis of many commercial and recreational fisheries (Lea et al. 1999, Williams et al. 2010), and declines in rockfish stocks led to the establishment of the largest marine protected areas in southern California, the Cowcod Conservation Areas (Thompson et al. 2017). Unfortunately, this shortcoming cannot be easily overcome through additional *I2S* barcoding. This is because rockfish are a recent and diverse radiation comprised of 110 species (Ingram and Kai 2014b) and *I2S* fails to resolve most *Sebastes* to species-level (Hyde and Vetter 2007, Yamamoto et al. 2017). Thus effective metabarcoding of *Sebastes* will require designing novel *Sebastes*-specific metabarcoding primers that target a more rapidly evolving region of the mitochondrial genome (e.g. *CytB*) (Thompson et al. 2017).

However, FishCARD includes 100% of all non-*Sebastes* nearshore species monitored by the Channel Islands National Kelp Forest Monitoring Program (n=80, Sprague et al., 2013), as well as by PISCO, the Partnership for Interdisciplinary Studies of Coastal Oceans (n=76; (Pondella II et al. 2015, Caselle et al. 2015). Further, there is now *I2S* reference sequence for 98 of the 100 most abundant ichthyoplankton species collected by the California Cooperative Oceanic Fisheries Investigation (CalCOFI) from the California Current between 1951-2019 (only Showy bristlemouth *Cyclothone signata* and Spotted barracudina *Arctozenus risso*) (Moser 1993). Moreover, in real world application, this reference barcode database assigned taxonomy to over 90% of vertebrate ASVs detecting a broad range of ecologically and commercially

important nearshore rocky reef species (Pondella II et al. 2019). As such, FishCARD represents an important genetic resource for coastal California marine metabarcoding monitoring efforts.

### *Limitations of MiFish primers*

High numbers of unidentified ASVs are a common feature of barcoding and metabarcoding studies (e.g. Leray & Knowlton, 2017). These unidentified ASVs are typically attributed to incomplete reference databases (Ransome et al. 2017, Curd et al. 2019, Schenekar et al. 2020) and/or novel biodiversity (Barber and Boyce 2006, Boussarie et al. 2018). However, given that FishCARD includes 82.8% of all California Current fishes, and the remaining 15.6% are mostly rare species unlikely to be found in a kelp forest, it was extremely surprising that half of all ASVs and a quarter of all sequences generated in our eDNA test datasets could not be assigned.

The vast majority of these sequences and ASVs did not belong to vertebrates, but instead uncultured marine bacteria, specifically matching to *16S*, rather than *12S* loci. Since mitochondria represent the capture of microbial endosymbionts by ancient eukaryotes (Roger et al. 2017) and that this capture occurred in the sea, it perhaps is not surprising that primers designed to target vertebrate *12S* might also capture marine prokaryotes. Similarly, the homology between vertebrate *12S* and prokaryotic and bacterial *16S* genes is well known (Crews and Attardi 1980) suggesting capturing microbial *16S* with vertebrate *12S* primers is also not surprising. However, this particular feature of the MiFish primer set was previously unreported, potentially impacting the interpretation of unidentified ASVs in fish metabarcoding studies.

These findings highlight the importance of accurate universal metabarcoding primer design, especially in outlining both target and non-target sequences. In the design of the MiFish Teleost *12S* primers, uncultured marine microbe *16S* sequences were not considered as potential

alternative targets for the primer set, resulting in the selection of a metabarcoding locus with a high degree of non-target amplification (Miya et al. 2015). This is an important finding for the marine vertebrate eDNA community which has recently converged on the MiFish *12S* primers (Closek et al., 2019; O'Donnell et al., 2017; Valsecchi et al., 2019; Yamahara et al., 2019) as these results suggest that the MiFish *12S* primer set will generate substantial quantities of non-target eDNA reads. At best, this non-target amplification will lead wasted sequencing effort, as every microbial sequence generated reduces the number of vertebrate sequences. At worst, it could result in incorrect interpretation of unidentified ASVs. This problem is of particular concern in environments with high relative abundance of marine bacterial communities and low relative abundance of vertebrate biomass such as in some pelagic midwater and deep-sea habitats where recent eDNA sample collection efforts have struggled to detect vertebrate sequences (K. Pitz personal communication).

#### *Towards improved metabarcoding*

FishCARD was designed to improve effectiveness of metabarcoding of California Current marine fishes. To further improve and expand the taxonomic coverage of the database, we generated a website that identifies species needing *12S* reference barcodes and provides the research community targets for additional barcoding efforts (<https://github.com/zjgold/FishCARD>). The ability to update and expand FishCARD will be especially important as climate change leads to range expansions of sub-tropical species that may become resident within the California Current Large Marine Ecosystem (Gentemann et al. 2017, Harvell et al. 2019, Sanford et al. 2019). The importance of expanding the database is highlighted by our detection of Finescale triggerfish, *Balistes polylepis*, in the eDNA samples, a

species that has only recently become more common off Santa Cruz Island and La Jolla since the 2014-2016 marine heatwave (B. Frable & S. McMillan, personal communication).

Additionally, while the MiFish Teleost and Elasmobranch *12S* loci are important targets for current marine metabarcoding studies, future efforts and different applications of marine metabarcoding will likely rely on additional barcoding targets. Recent efforts have found success multiplexing *COI* and *16S* loci simultaneously, providing more species-level identifications than either marker alone and demonstrating complimentary genetic loci can improve metabarcoding assignments (Duke & Burton, 2020). Future efforts to develop rapid and affordable multi-loci barcoding and mitogenomic tools will provide greater resources for marine metabarcoding and population genomic efforts (Coissac et al. 2016). As these new barcode loci are developed (e.g. *Sebastes*-specific barcodes), FishCARD can be expanded to include these loci. Additionally, resources like the SIO Marine Vertebrates Collection will continue to provide important voucher specimens for advancing marine molecular ecology resources as they accession new material.

Here we demonstrate that FishCARD provides an important genetic resource for California Current marine metabarcoding efforts, improving the accuracy and effectiveness of this important and growing research tool. The development of robust and complete reference databases dramatically improves the accuracy of species-level taxonomic assignments, in turn enhancing the efficacy and applicability of these tools for marine biomonitoring. This tool dramatically improves fish eDNA metabarcoding efforts in the California Current Large Marine Ecosystem and provides marine resource managers and researchers an important tool for surveying and monitoring marine fish communities using eDNA.

## CHAPTER 2

### **eDNA Metabarcoding as a Biomonitoring Tool for Marine Protected Areas**

#### **Abstract**

Monitoring of marine protected areas (MPAs) is essential for marine ecosystem management. Current protocols rely heavily on SCUBA-based visual observations that are costly and time consuming, limiting the scope of MPA monitoring. Environmental DNA (eDNA) metabarcoding is a promising alternative for marine ecosystem monitoring, but more direct comparisons to traditional visual surveys are needed to understand the strengths and limitations of each approach. This study compares fish communities inside and outside the Scorpion State Marine Reserve off Santa Cruz Island, CA using eDNA metabarcoding and underwater visual census surveys. Results from eDNA captured 76% (19/25) of fish species and 95% (19/20) of fish genera observed during pairwise underwater visual census. Species missed by eDNA were due to the inability of MiFish *12S* barcodes to differentiate species of rockfishes (*Sebastes*, n=4) or low site occupancy rates of crevice-dwelling *Lythrypnus* gobies. However, eDNA detected an additional 30 fish species not recorded in paired visual surveys, but previously reported from prior visual surveys, highlighting the sensitivity of eDNA. Significant variation in eDNA signatures by location (50m) and site (~1000m) demonstrates the sensitivity of eDNA to address key questions such as community composition inside and outside MPAs. Interestingly, eDNA results recorded higher species richness outside the MPA while visual surveys observed the opposite pattern. This paradoxical result is likely caused by swamping effects of high fish abundance in MPAs that reduce detection probabilities of pelagic, sandy bottom, and intertidal

taxa. Results demonstrate the utility of eDNA metabarcoding for monitoring MPAs, providing an important complementary tool to visual monitoring methods, helping expand MPA monitoring activities across space, time, and depth.

## **Introduction**

Marine Protected Areas (MPAs) promote sustainability of marine ecosystems and the ecological goods and services they provide (Edgar et al. 2007, 2014, Claudet et al. 2010). However, ensuring MPA effectiveness requires regular monitoring to document that local ecosystem health is stable or improving (Edgar et al. 2014). MPA monitoring also provides an essential opportunity to assess the impact of management practices, allowing resource managers to adjust management plans as required (Edgar et al. 2014).

Current MPA monitoring protocols focus on assessing the diversity and abundance of fish and benthic invertebrates, as well as community trophic structure (Usseglio 2015). Much of this assessment is based on underwater visual census surveys conducted on SCUBA (Usseglio 2015), a method with notable limitations. SCUBA-based surveys are inherently costly, and time and labor intensive (Lessios 1996, Murphy and Jenkins 2010). For example, to survey 33 sites within the Channel Islands National Park once per year, the National Park Service Kelp Forest Monitoring Program spends over \$470,000 on ~1,000 hours of dive time, ~2,000 hours of data entry and quality control (Sprague et al. 2013). Furthermore, SCUBA-based surveys are constrained by weather, diving conditions, and personnel (J. Sprague per. obs., 2020), and can require extended and repeated dives to accurately document marine communities (Denoble et al. 2011) that place divers at risk for dive-related injuries. SCUBA surveys can also introduce significant observer bias, as fish react differently to divers, particularly inside and outside of MPAs, potentially impacting survey results (Lindfield et al. 2014, Pereira et al. 2016).

Given the above logistical and methodological constraints, MPA monitoring efforts are largely limited to the most economically or ecologically important taxa as proxies for ecosystem health and to relatively shallow depths (e.g. <30m) (Pondella II et al. 2015). Moreover, examining a predetermined subset of community diversity potentially excludes crucial functional groups, biasing ecosystem assessment (Willis 2001, Edgar et al. 2004, Bernard et al. 2013). Combined, these limitations restrict the scope, scale, and frequency of visual surveys, limiting the utility of SCUBA-based MPA surveys to quantify species diversity and trophic structure, (Usseglio 2015), data essential for assessing MPA effectiveness.

One promising new approach for assessing and monitoring marine ecosystems is environmental DNA, or “eDNA”, a technique based on isolation and sequencing of freely associated DNA from soil or water samples (Taberlet et al. 2012a). Through metabarcoding and high-throughput next generation sequencing, eDNA can broadly survey community biodiversity in a rapid, repeatable, and affordable manner (Deiner et al. 2017). As such, eDNA is ideally suited to intensive biodiversity monitoring programs, such as those required for MPAs (Kelly et al. 2014b, Bohmann et al. 2014).

eDNA has some key advantages over traditional SCUBA-based survey methods. First, eDNA can capture a wide diversity of marine vertebrate and invertebrate taxa, frequently detecting more species than traditional fish survey methods (Thomsen et al. 2012, 2016, Port et al. 2015, Yamamoto et al. 2016). Second, eDNA detects rare and cryptic species that are frequently overlooked or ignored in traditional survey methods (Willis 2001, Port et al. 2015, Valentini et al. 2016), including both endangered and invasive species (Dejean et al. 2012). Third, eDNA collection is relatively simple, requiring only small volumes of seawater (< 3L) and simple filtering techniques, allowing sampling by individuals with limited training, even in



remote locations (Miya et al. 2016). Forth, because eDNA doesn't require diving, there are significant worker safety advantages. Lastly, eDNA is affordable (e.g. ~\$50/sample) and has the potential for automation, allowing for remote sample collection and high throughput autonomous lab processing (Good et al. 2018, Yamahara et al. 2019).

Despite these advantages, eDNA also has limitations. Of particular concern is PCR bias that can result in preferential amplification of particular taxa, biasing read abundances (Kanagawa 2003, Pawluczyk et al. 2015, Krehenwinkel et al. 2017, Kelly et al. 2019). Additionally, detection probabilities can be influenced by species specific eDNA generation and degradation rates (Deiner et al. 2017), an issue potentially further complicated by the transport of eDNA on ocean currents (Barnes and Turner 2016, Andruszkiewicz et al. 2019). In addition, primer design, bioinformatic, and reference database limitations can also affect the accuracy of taxonomic assignment from eDNA (Deiner et al. 2017, Alberdi et al. 2018).

As a relatively new method, the biases in eDNA metabarcoding are not as well characterized as visual surveys. Empirical studies indicate that impacts of PCR bias can be mitigated by technical replicates and site occupancy modelling (Alberdi et al. 2018, Chambert et al. 2018, Doi et al. 2019). Similarly, because eDNA signals decay relatively rapidly (e.g. hours to days; Yamamoto et al. 2016; Andruszkiewicz et al. 2017; Jo et al. 2017; Kelly et al. 2018; Murakami et al. 2019), eDNA signatures are surprisingly stable (Kelly et al. 2018). As such, eDNA holds tremendous promise for monitoring marine ecosystems, but realizing that promise requires a better understanding of how visual surveys and eDNA metabarcoding approaches compare in direct field applications.

Established in 2012, the Southern California MPA Network is partially monitored by the National Park Service Kelp Forest Monitoring Program, which conducts visual monitoring

surveys of a fraction of local fish and benthic invertebrates species (Allen and Horn 2006, Sprague et al. 2013). Further, only 33 of the >1000 Channel Island reefs are surveyed, and just once per year (Sprague et al. 2013), missing the seasonal dynamics in the variable Southern California Bight, limiting the scope and scale of assessment (Steneck et al. 2002, Schiel and Foster 2015). While born of logistical necessity, the spatial and temporal limits of this survey protocol makes accurately assessing the health of this MPA network difficult (Hamilton et al. 2010, Caselle et al. 2015, Gill et al. 2017, Nickols et al. 2019) and suggests the need for new approaches that produce data on broader taxonomic, spatial and temporal scales.

In this study, we conducted comparisons of eDNA metabarcoding and visual survey protocols performed by the National Park Service Kelp Forest Monitoring Program. We conducted this comparison in the Scorpion State Marine Reserve off Eastern Santa Cruz Island in Southern California, CA, USA examining fish communities to test the efficacy of eDNA for MPA monitoring and to better understand the advantages and shortcomings of this method.

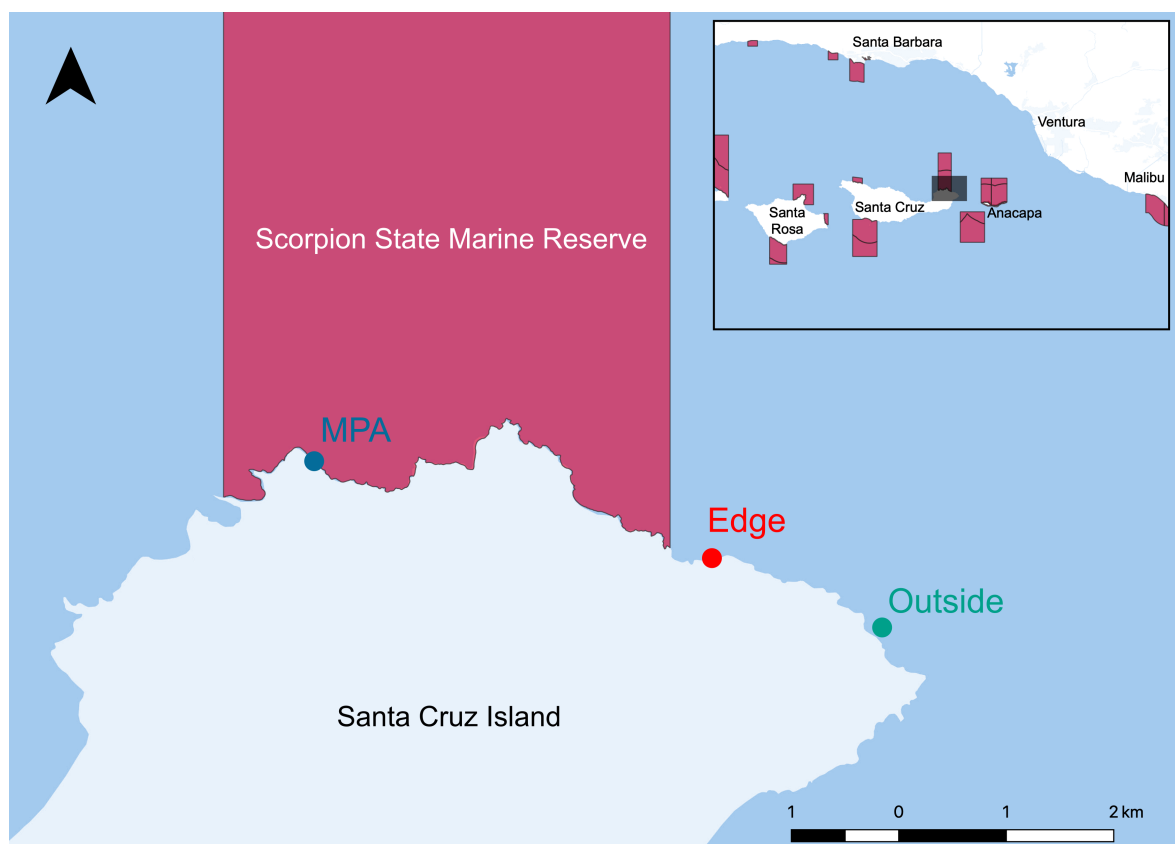
## **Materials and Methods**

### *Sample Collection*

We conducted our study at Scorpion State Marine Reserve within the Channel Islands National Marine Sanctuary. To determine the degree to which eDNA could capture documented differences inside and outside this MPA (Sprague et al. 2013, Pondella II et al. 2015), we sampled three sites: 1) inside the MPA, 2) outside but adjacent (<0.5km) to the MPA (“edge site”), and 3) 2.3km outside the MPA boundary (“outside site”; Figure 2-1). At each of these three sites, we sampled along a 100m transect, using a GPS to ensure these transects overlapped with fixed 100m transects used by the Kelp Forest Monitoring Program for visual monitoring. We collected three

replicate 1L water samples from three locations on each transect, totaling 9 spatially structured replicates per site. Due to vessel maintenance challenges, each site was sampled on a different day with a maximum of 72 hours between sampling events. eDNA samples were collected between August 9<sup>th</sup>, 2017 and August 11<sup>th</sup>, 2017.

**Figure 2-1.** Map of Scorpion State Marine Reserve off Santa Cruz Island, CA, USA.



We collected seawater samples from 10m below the surface and 1m above the benthos using a 4L Niskin bottle deployed from the UCLA RV Kodiak (Thomsen et al. 2016). From each Niskin deployment, we transferred a single liter of seawater to an enteral feeding pouch (hereafter “pouch”), which we then hung to facilitate gravity filtration through a sterile 0.22  $\mu$ m Sterivex cartridge filter (MilliporeSigma, Burlington, MA, USA) in the field (Miya et al. 2016). Additionally, we processed three field blanks as a negative control that consisted of 1L of distilled

water following the method above (Goldberg et al. 2016). Finally, we dried eDNA containing Sterivex filters using a 3mL syringe to push through all remaining sea water, and then capped and stored the filters at -20°C for DNA laboratory work back at UCLA (Miya et al. 2015).

### *DNA Extraction and Library Preparation*

We extracted eDNA from the Sterivex cartridge using the DNAeasy Tissue and Blood Kit (Qiagen Inc., Germantown, MD) following modifications of Spens et al. (2017), directly adding proteinase K and ATL buffer inside the filter cartridge before an overnight incubation. We PCR amplified the extracted eDNA using the MiFish Universal Teleost *I2S* primer (Miya et al. 2015) with Nextera modifications following Curd et al. (2019) (See Appendix B – Supplemental Methods). All PCRs included a negative control where molecular grade water replaced the DNA extraction. For positive controls, we used DNA extractions of Grass carp (*Ctenopharyngodon idella*) and Atlantic salmon (*Salmo salar*), both non-native to California. To ensure amplification success and correct product size, we electrophoresed all PCR products on 2% agarose stained with SybrGreen.

We prepared PCR products for sequencing by pooling 5µL of triplicate PCR reactions (“technical replicates”), cleaning the pooled samples using Serapure magnetic beads (Faircloth and Glenn 2014) and then quantifying their concentrations using the Quant-iT™ dsDNA Assay Kit (Thermofisher Scientific, Waltham, MA, USA) on a Victor3 plate reader (Perkin Elmer Waltham, MA, USA). We prepared sample DNA libraries using indexes from both the Nextera Index A and D Kit (Illumina, San Diego, CA, USA) and KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Wilmington, MA, USA) following Curd et al. (2019) (See Appendix B – Supplemental Methods). We electrophoresed all indexed PCR products on 2% agarose gels to confirm correct product size, and bead cleaned and quantified the resulting libraries as described above. Finally, we pooled

Indexed libraries in equimolar concentration, and sequenced the libraries on a MiSeq PE 2x300bp at the Technology Center for Genomics & Bioinformatics (University of California- Los Angeles, CA, USA), using Reagent Kit V3 with 20% PhiX added to all sequencing runs.

### *Bioinformatics*

To determine community composition, we used the *Anacapa Toolkit* to conduct quality control, amplicon sequence variant (ASV) parsing, and taxonomic assignment using user-generated custom reference databases (Curd et al. 2019). We processed sequences using the default parameters and assigned taxonomy using two reference databases. We first assigned taxonomy using the FishCARD California fish specific reference database (Gold *et al.* in prep). Second, we used the *CRUX*-generated *I2S* reference database supplemented with FishCARD reference sequences to assign taxonomy using all available *I2S* reference barcodes to identify any non-fish taxa. We transferred the resulting species community tables into *R* for subsequent downstream data analysis (Team 2014).

Prior to alpha and beta diversity analyses, the raw ASV community table was decontaminated to ensure that potential field contamination, lab contamination, and sequence index hopping did not influence the results (Goldberg et al. 2016, Costello et al. 2018). Decontamination followed Kelly et al. (2018) and McKnight et al. (2019) (See Appendix B – Supplemental Methods) and implemented a three step protocol: 1) identifying and removing sequences arising from index hopping, 2) identifying and removing sequences from negative controls, and 3) conducting site occupancy modeling to identify true positive detections (Schmidt et al. 2013, Ficetola et al. 2016, Lahoz-Monfort et al. 2016). For site occupancy models we accepted all species with an estimated occurrence (detection) probability over 75% and occurred in at least two replicates across the 9 spatially structured replicates taken at a given site. Following the appropriate decontaminations, we

transformed all read counts into an eDNA index for beta-diversity statistics (Kelly et al. 2019).

Although the MiFish Universal Teleost primer set is designed to amplify teleost fishes, the primers amplify broad range of marine fish and vertebrate taxa (Miya et al. 2015, Valsecchi et al. 2019). As such, all non-fish species (mammals and birds) were removed prior to final analyses.

### *eDNA Data Analysis*

We computed alpha diversity statistics for each of the three sites to quantify site overlap and dissimilarities of fish communities inside and outside the MPA (McMurdie and Holmes 2013). Total species richness for each site was then compared, using an Analysis of Variance (ANOVA) to test for alpha diversity differences (Oksanen et al. 2013). We tested assumptions of the ANOVA using Levine's test for homogeneity of dispersions (Oksanen et al. 2013).

To determine whether our eDNA sampling design was sufficient to fully capture fish community diversity, we created species rarefaction curves using the iNext package which has demonstrated higher accuracy in estimating species richness from species occurrence data (Hsieh et al. 2016). Species coverage estimates, the proportion of species detected from the estimated total site diversity, were then compared between each site.

To compare the relative effectiveness of eDNA methods for detecting all species and common species, we calculated species coverage estimates with and without site occupancy modeling. We also compared species coverage estimates using all 3 1L replicates taken at 3 locations along a 100m transect (n=9) as well as only 3 1L biological replicates (n=3), a frequently employed sampling design used in marine eDNA studies. We ran a piecewise regression analysis to identify breakpoints in the rate of species diversity found per sample collected using the *R* packaged *segmented* (Muggeo 2008).

To test for differences among fish communities, we calculated Bray-Curtis similarity distances on the eDNA index scores between all samples (Kelly et al. 2018). Specifically, we tested for the difference in community similarity variance between our three sites using an *adonis* PERMANOVA (Oksanen et al. 2013), followed by a companion multivariate homogeneity of group dispersions test (BETADISPER) (Oksanen et al. 2013). Both the PERMANOVA and BETADISPER were run using the following model: eDNA Index ~ Site + Location. We also visualized community beta diversity using non-metric multidimensional scaling (NMDS) (Oksanen et al. 2013).

To further investigate which species were driving eDNA community differences among sites, we conducted constrained analysis of principle components (CAP) (Oksanen et al. 2013). We then identified the species that explained the greatest degree of difference across the two principle components.

### *Visual Underwater Census Methods*

To assess fish communities using underwater visual census techniques, SCUBA divers from the National Park Service Kelp Forest Monitoring Program followed standard survey protocols following Sprague et al. (2013) and Gillett et al. (2012). Underwater visual surveys were conducted on the following days: MPA site on September, 27<sup>th</sup>, 2017; edge site on August 7<sup>th</sup>, 2017, and outside site on June 5<sup>th</sup>, 2017. These protocols include three survey types: visual fish transects, roving diver fish counts, and 1m quadrat transects. The visual fish transects targeted 13 indicator species of fish on visual fish transects. Roving diver fish count surveys record any species observed along the transect. The 1m quadrat transects records three small demersal species of fish: *Lythrypnus dalli*, *Lythrypnus zebra*, and *Alloclinus holderi*. All visual surveys occurred along a permanent 100m transect at each site (See Appendix B – Supplemental Methods).

## *Comparison of eDNA and Visual Underwater Census Methods*

We compared species detected by eDNA and underwater visual census approaches across corresponding transects at each site. We identified core taxa that were shared across all sites for a given method. In addition, we identified species that eDNA methods failed to detect but were observed in visual census surveys and vice versa. We note that given the few numbers of sites (n=3) we were unable to robustly compare abundance estimates between methods.

## **Results**

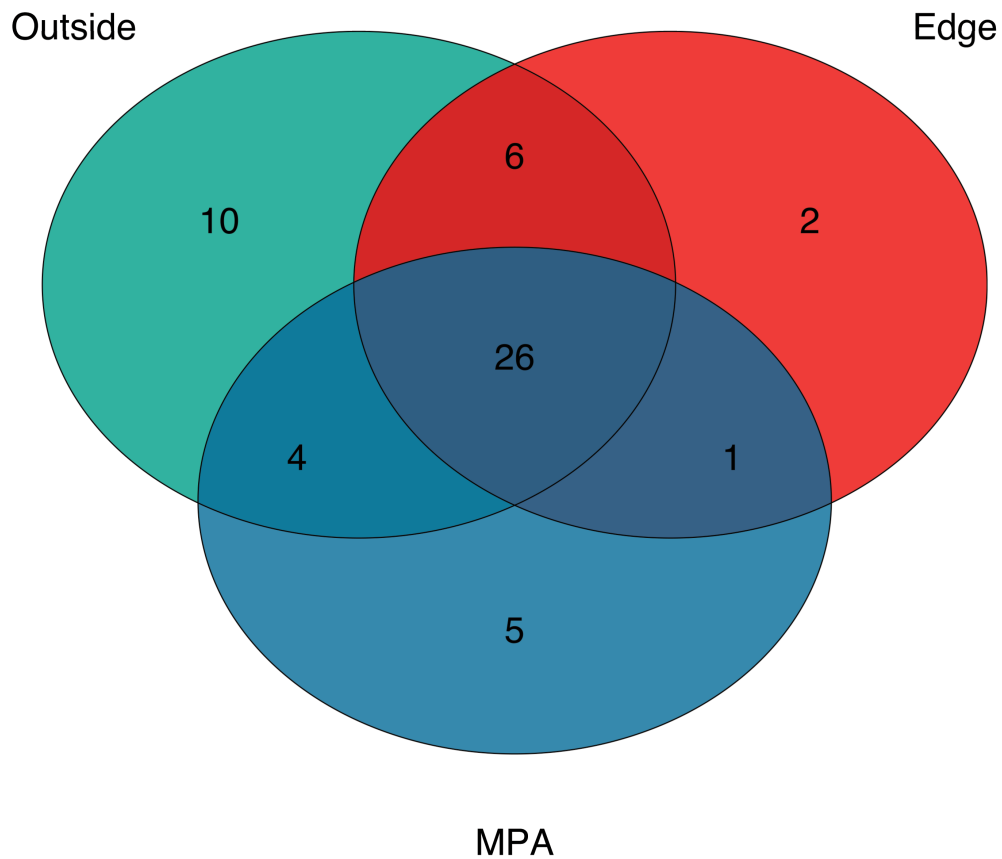
### *eDNA Results*

We generated over 4 million reads that passed Illumina MiSeq quality control filters. The *Anacapa Toolkit* identified 2,906 ASVs from 3,091,063 reads representing 27 samples and 8 controls. After the second decontamination step, however, totals reduced to 931 ASVs and 2.35 million reads (Supplemental Tables 2-S1 through 2-S3).

Combined, eDNA metabarcoding successfully detected 54 fish taxa, representing 50 unique species, 48 genera, 34 families, and 2 classes (Supplemental Tables 2-S1 through 2-S3). eDNA detected 35 species within the MPA, 34 at the edge, and 42 species outside the MPA. The three sites shared a core group of 26 taxa including bony fish and one species of ray (Figure 2-2) (Supplemental Table 2-S4). Of these taxa, 15 species are associated with rocky reef habitat, 5 species are associated with sandy bottom habitat, 4 species are pelagic-neritic, and 2 species are pelagic-oceanic.



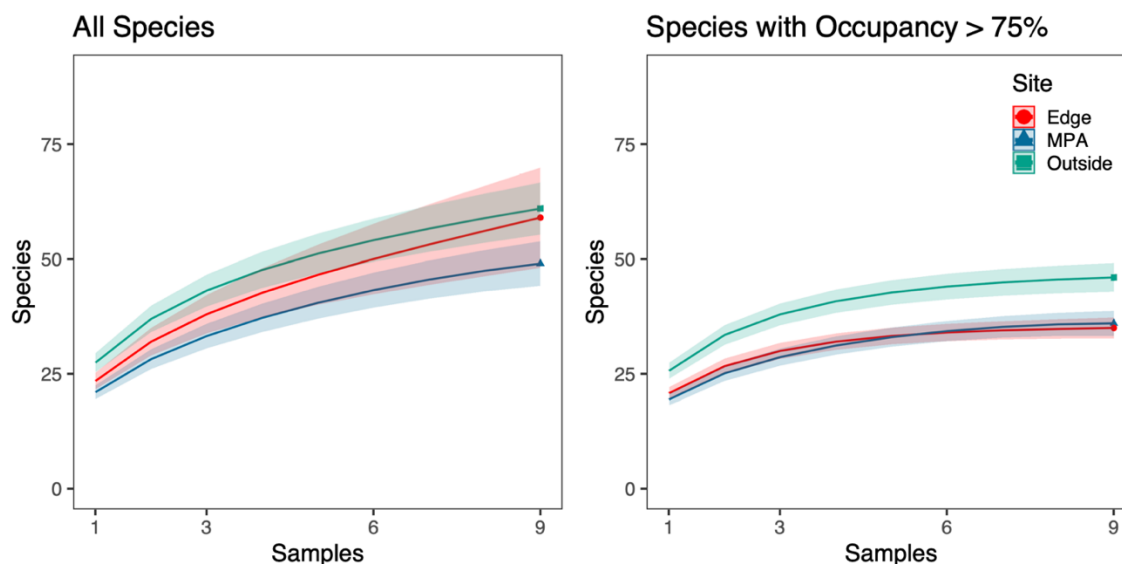
**Figure 2-2.** Venn Diagram of Fish Species Detected with eDNA



Species rarefaction curves showed that sampling at each site (n=9) was insufficient to capture all species diversity (Figure 2-3). Sample coverage estimates from eDNA results before filtering by site occupancy modeling filters were 94.0%, 88.0%, and 92.9% for the MPA, edge, and outside sites, respectively. Coverage estimates dropped to 81.0%, 80.0%, 83.6% for the MPA, edge, and outside sites, respectively, when only 3 1L samples were used. Piecewise regression analysis showed a transition from exponential to linear increase in species detected per replicate between 3 and 4 replicate water samples per site (3.36-3.53) with subsequent diminishing sample coverage returns with the addition of more samples. In contrast, species diversity was near saturated (>99.0%) when applying a site occupancy rate above 75% and using

3 1L replicates taken at 3 locations along a 100m transect. However, using only three samples, sample coverage dropped to 87.1%, 90.3%, 88.9% for the MPA, edge, and outside sites, respectively.

**Figure 2-3. Species Rarefaction Curves.** **a)** Species rarefaction curves for all fishes found at each site across 3 1L replicates taken at 3 locations along a 100m transect. **b)** Species rarefaction curves for fish species with occupancy rates above 75% found at each site across 3 1L replicates taken at 3 locations along a 100m transect. Sample coverage estimates were higher for species with occupancy rates above 75% (100%) than for all species (85.8%-93.1%). For species with occupancy rates above 75% sample coverage estimates ranged from 89.3-91.1% for only 3 1L replicates.

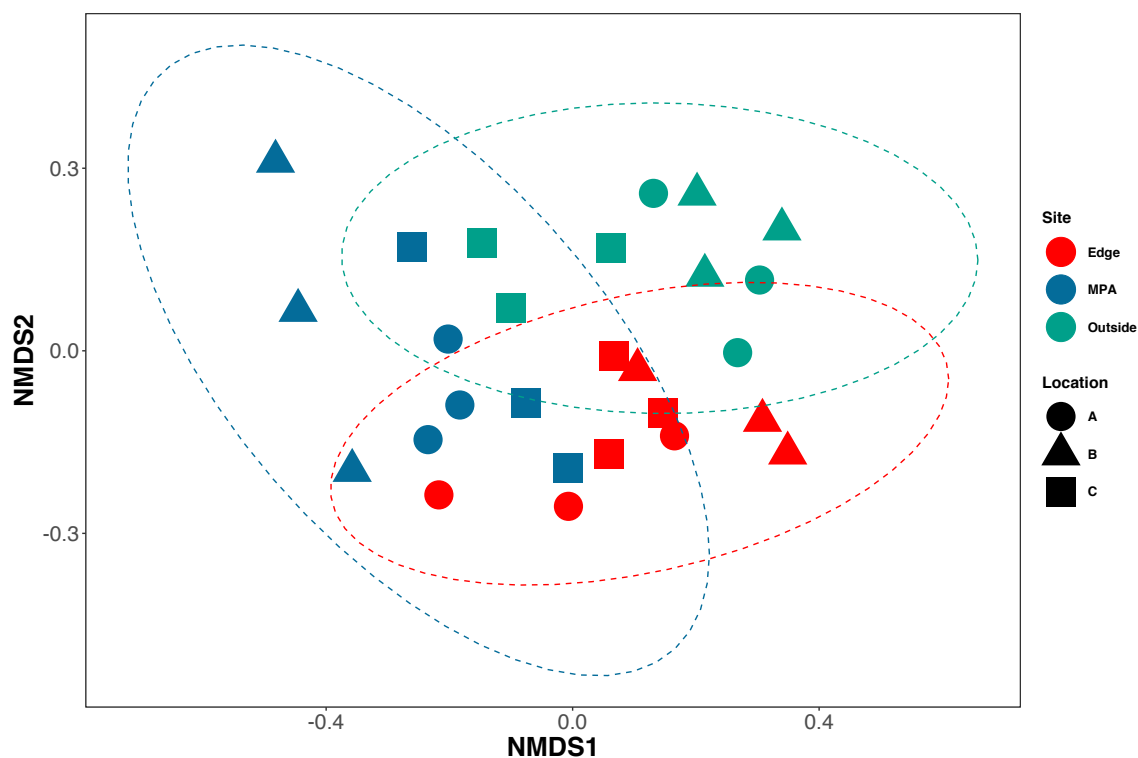


Analyses showed a significant difference in the total number of observed species across sites, with the site outside the MPA having significantly higher diversity than both the edge and MPA sites (ANOVA,  $p < 0.001$ , Levine's test  $p > 0.5$ ). Observed species differences between sites were partially driven by the presence of non-rocky reef taxa (46.4%, 13/28), primarily pelagic,

mobile, sandy bottom, and intertidal species. Moreover, there were also significant differences in fish communities among the three sites as well as among the three sampling locations along each of the three transects (PERMANOVA  $p < 0.001$ , betadisper  $p > 0.05$ ). Location along the transect explained 26.4% of the total variance while site (e.g. inside, edge and outside MPA) explained 19.0% of the total variance; 54.5% of the total variance was unexplained.

NMDS ordination showed weak clustering of samples by both location and site (NMDS, Stress 0.20; Figure 2-4).

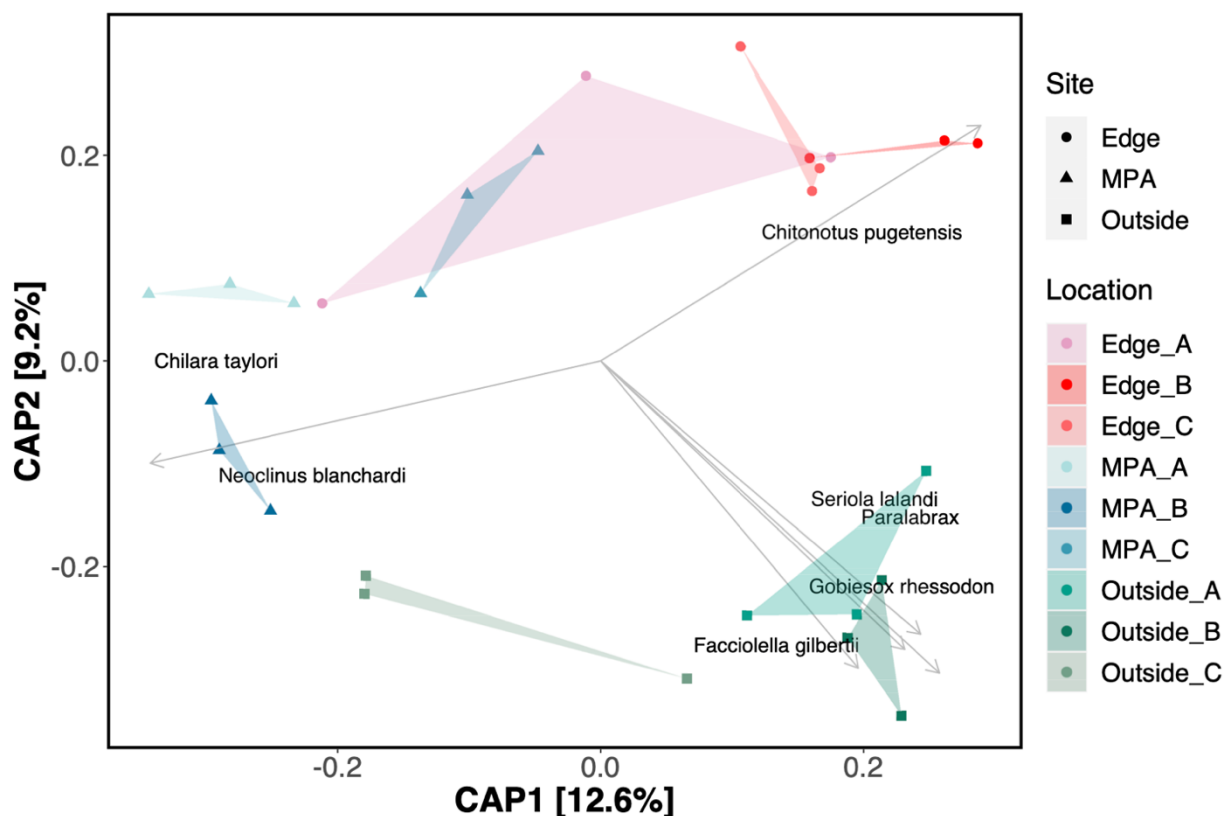
**Figure 2-4.** NMDS of Bray-Curtis Dissimilarities. Bray-Curtis dissimilarities were calculated between all samples using only species with occupancy rates over 75%. Samples from Sites (colors) and locations (shapes) are similar to each other (NMDS, Stress = 0.20).



Constrained analysis of principle components (CAP) found significant differences in species assemblages between samples collected at different sites and locations (CAP,  $p < 0.001$ ) (Figure 2-5), further indicating difference in eDNA signatures across sites and locations. CAP analysis identified 7 taxa with the strongest differences between sites. The MPA site had higher eDNA index scores of Kelp perch (*Brachyistius frenatus*), Sarcastic fringehead (*Neoclinus blanchardi*), and Spotted cusk-eel (*Chilara taylori*). The edge site had higher index scores of Roughback sculpin (*Chitonotus pugetensis*). The site outside the MPA had higher index scores of Yellowtail

amberjack (*Seriola lalandi*), Sand bass sp. (*Paralabrax* sp.), and Dog-faced witch eel (*Facciolella gilberti*).

**Figure 2-5.** Constrained Analysis of Principle Components (CAP) Ordination. Bray-Curtis dissimilarities were calculated between all samples using only species with occupancy rates over 75%. Site and locations within sites are significantly more similar to each other (CAP,  $p < 0.001$ ). Sites (shapes) and Locations (colors) are plotted against CAP1 and CAP2 axes. Arrows correspond to direction and strength (length) of each species. Only the top 7 species with CAP distances greater than 0.35 were plotted.

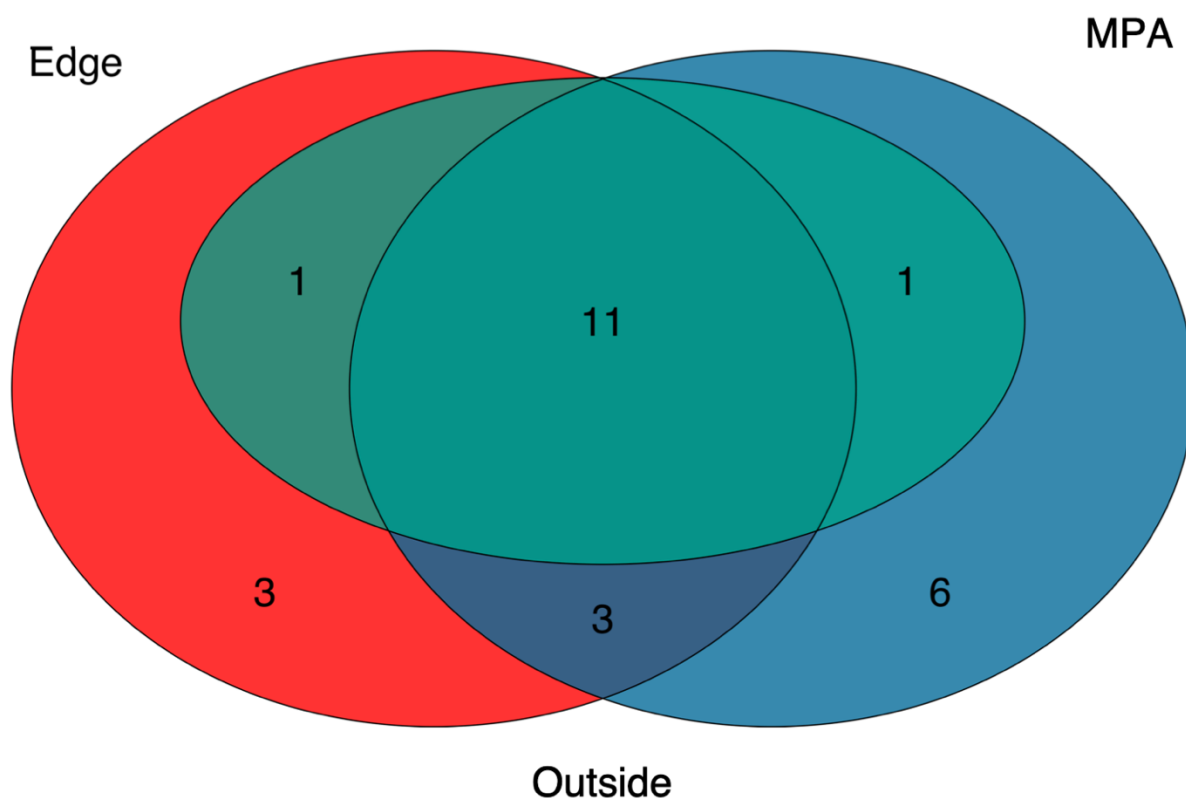


### Visual Census Surveys Results

Across all three sites, 25 bony fish species were recorded using underwater visual censuses, representing 20 genera, 13 families, and 1 class (Figure 2-6) (Supplemental Table 2-S5), 11 of

which were shared across all three sites (Supplemental Table 2-S6). Within the MPA site, visual census methods detected 21 unique species, 18 genera, and 11 families. At the edge site visual census methods detected 18 species, 16 genera, 11 families, and four classes. Lastly, at the outside site visual census methods detected 13 species, 13 genera, 10 families, and four classes. Of all taxa observed in visual census methods, 24 species are associated with rocky reef habitat and 1 species is pelagic-neritic. The pelagic-neritic species, top smelt (*Atherinops affinis*), was only found in the MPA site.

**Figure 2-6.** Venn Diagram of Species Observed from Visual SCUBA Surveys.



On average, roving diver fish counts recorded 17.6 species per replicate survey (Range: 10-22). Visual fish counts recorded an average 7.8 species per replicate survey out of the 13 indicator species (Range: 5-10). 1m quadrats recorded an average 2.3 species of 3 target species (Range: 1-3).

### *Comparison of eDNA and Visual Census Surveys*

eDNA detected 76% (19 out of 25) of species observed during National Park Service visual transect surveys (Supplemental Table 2-S5 & 2-S6). eDNA failed to resolve *Lythrypnus dalli*, *L. zebra*, *Sebastes atrovirens*, *S. auriculatus*, *S. chrysomelas*, and *S. serranoides* to species level. At the genus level, eDNA performed markedly better recovering 95% (19 out of 20) of genera observed during under water censuses. The remaining genus *Lythrypnus* was detected prior to site occupancy modeling but occurred in only one replicate at two separate sites.

In addition to the above, eDNA recovered 31 species that were not recorded during the visual censuses conducted by the National Park Service. Of these, 30 were native California fish species previously observed and recorded in Kelp Forest Monitoring Program surveys (Supplemental Table 2-S7), but not during our paired surveys. In addition, eDNA detected the California native Dog-faced witch eel (*Faciolella gilberti*) that had not previously been observed by the Kelp Forest Monitoring Program.

There were few conspicuous differences in species observed across sites, with visual census results identifying 11 common taxa across all sites (Supplemental Table 2-S6). Of these, 10 were also found to be common across all sites using eDNA methods with one species (*Lythrypnus dalli*) not detected by eDNA. Species richness from visual census data showed that fish diversity was highest within MPA (n=21), lowest outside the MPA (n=13) and intermediate (n=18) on the edge of the MPA, while eDNA had the opposite pattern.

## Discussion

Results demonstrate the power of for detecting a broad range of fish biodiversity in California kelp forest ecosystems, providing more detailed species inventories needed for marine ecosystem monitoring (Port et al. 2015, Curd et al. 2019). Moreover, eDNA was able to detect significant differences in fish communities inside, on the edge of, and outside of the Scorpion State Marine Reserve, even though the closest sites were no more than 500m apart. Even within each of these sampling sites, eDNA distinguished among sample locations separated by only 50m, highlighting the sensitivity of eDNA in capturing local fish communities, and matching previous studies showing fine-scale spatial resolution of eDNA signatures (Port et al. 2015, Murakami et al. 2019).

Importantly, eDNA captured 76% of fish diversity observed during visual surveys, despite species rarefaction indicating insufficient sampling. In total, eDNA only failed to identify 6 of 25 fish species observed during visual surveys, the majority of these being rockfish (*Sebastes*), a taxon that *12S* barcoding cannot distinguish to species (Closek et al. 2019). However eDNA provided data on 30 additional fish taxa not recorded when implementing Kelp Forest Monitoring Program visual monitoring protocols (Sprague et al. 2013), highlighting an important advantage of eDNA. Because sampling can be obtained easily and economically, eDNA could allow for more frequent monitoring, expanding the scope of MPA monitoring programs while providing greater personnel safety.

### *The utility of eDNA for MPA monitoring*

Despite a limited sampling design and the inability of our *12S* barcode to distinguish species of rockfish and gobies, eDNA largely recovered the same taxa observed in visual census surveys. This strong concordance likely stems from high eDNA detection probabilities lasting only a few



hours (Murakami et al. 2019), such that eDNA captures marine communities that were recently present (Barnes and Turner 2016). This similarity among eDNA and visual surveys is even more remarkable given that eDNA and visual surveys were taken months apart, a result that strongly suggests that fish diversity captured by eDNA is truly representative of fish communities and their differences inside and outside the Scorpion State Marine Reserve (Kelly et al. 2018).

In addition to detecting fish recorded in visual surveys, eDNA recorded an addition 30 species not recorded from visual surveys but have been previously reported in other Kelp Forest Monitoring Program surveys (Supplemental Table 2-S7). Importantly, these taxa included species of significant management concern such as IUCN red-listed Giant black seabass (*Stereolepis gigas*) and important commercial targets like Yellowtail amberjack (*Seriola lalandi*). Additionally, although we focused on fishes, our eDNA data included elasmobranchs, marine mammals, and marine birds, taxa that play important roles in nearshore rocky reef ecosystems, but can be difficult to survey and monitor (Boussarie et al. 2018, Valsecchi et al. 2019). The expanded taxonomic coverage and the ability to detect rare or hard to observe taxa is a significant advantage of eDNA over traditional visual surveys, expanding the scope of MPA monitoring by capturing entire communities rather than a selected subset of taxa.

Key to MPA monitoring is the ability distinguish among communities inside and outside of the MPA. Not only did eDNA detect significant differences inside and outside the MPA, it could also differentiate among samples taken 50m apart. This result adds to a growing literature that shows the fate and transport of eDNA in marine environments is relatively limited in space and time (Yamamoto et al. 2016, O'Donnell et al. 2017, Tillotson et al. 2018, Murakami et al. 2019), and highlights the suitability of eDNA for comparing inside and outside of even relatively small MPAs (Port et al. 2015).

While eDNA found significant differences inside and outside of the MPA and provided data on more taxa than visual survey methods, it did it for a fraction of the cost and effort. Roving fish diver counts, the most similar visual survey to eDNA monitoring methods, costs the Kelp Forest Monitoring Program ~\$1,380 per site (Supplemental Table 2-S8). In contrast, the eDNA sampling design employed in this study including materials, labor, and transportation was ~\$600 per site (Supplemental Table 2-S9)—and 25% of this total was just transportation. Moreover, total costs could have been significantly reduced by sampling in one day, which was not possible due to boat mechanical issues. Further cost efficiencies can come from automating lab methods and conducting sequencing in house (Deiner et al. 2017).

In addition to the above, eDNA has other significant advantages. It can potentially detect invasive species, even when rare (Klymus et al. 2017). Sequence data from eDNA provides an annual snapshot of standing genetic diversity, providing the ability to monitor changes over time (Kelly et al. 2014b). Similarly, in species with population structure, eDNA could provide evidence of range shifts associated with climate change (Sanford et al. 2019). Importantly, given eDNA metabarcoding samples can be preserved and archived, eDNA samples can be reanalyzed in the future with improved metabarcoding methods to answer additional hypotheses and environmental monitoring goals (Bohmann et al. 2014, Deck et al. 2017). Combined, the above advantages of eDNA suggest that even if eDNA metabarcoding isn't viewed as a full replacement for visual surveys, the power of this method, its ease of sampling and affordability argue for using eDNA as a critically important complementary tool to greatly expand current monitoring activities.

### *Limitations and Caveats of eDNA*

Although this and other studies highlight the promise of eDNA for monitoring marine ecosystems (Kelly et al. 2014b, Bakker et al. 2017, Stat et al. 2017), there are also important

limitations. One key limitation is the lack of universal barcode loci. Four of the six undetected species in this study were rockfish in the genus *Sebastes*. While the MiFish 12S metabarcoding primers have broad utility in vertebrates, this region is highly conserved in rockfishes, a very recent adaptive radiation (Hyde and Vetter 2007, Miya et al. 2015), resulting in the inability to distinguish among rockfish ASVs. Identifying rockfish to species using eDNA approaches is critical for MPA monitoring efforts in California as *Sebastes* are important for commercial and recreational fisheries (Lea et al. 1999, Williams et al. 2010), exhibiting a wide array of functional and ecological diversity in nearshore ecosystems (Mangel et al. 2007, Ingram 2011). Novel *Sebastes*-specific metabarcoding primers targeting a more variable region of the mitochondria (e.g. *CytB*) are needed (Thompson et al. 2017).

In addition, eDNA metabarcoding approaches failed to detect two gobies, *Lythrypnus dalli* and *L. zebra*. Previous efforts to barcode *L. dalli* for the FishCARD reference database found two insertions not found in any other native California goby, including the sister species *L. zebra* (Gold *et al.* in prep). This result suggests that primer mismatch may have limited the amplification and detection of some *L. dalli* in our eDNA samples. Interestingly, we were able to amplify *L. zebra* which is genetically similar to *L. dalli* (Maxfield et al. 2012), albeit in only one replicate at two sites.

Alternatively, the eDNA methods employed here may not be ideally suited for small, crevice-dwelling fish species such as gobies (Hartney 1989). Species of *Lythrypnus* rarely leave the reef boundary layer (Behrems 1984). As such their eDNA maybe entrained close to the reef, resulting in hyper-spatial variability of eDNA signatures (Port et al. 2015). More work is necessary to determine whether eDNA can reliably detect species living in interstitial reef habitat. This limitation, however, is not unique to eDNA as the Kelp Forest Monitoring Program

employs 1m quadrat surveys, specifically designed to capture these hard to see taxa. Likewise, eDNA surveys that specifically sample within the boundary layer, not 1m above the reef, may be needed to survey crevice-dwelling species.

Another limitation of eDNA is standardizing processing techniques, including the spatial design of field sampling, number of replicates, and sequencing depth (Deiner et al. 2015, Calderón-Sanou et al. 2019, Doi et al. 2019, Jeunen et al. 2019, Kelly et al. 2019). The three replicate water samples taken from a single location and time recovered only 81.5% of the species present based on modeled species coverage estimates of species with at least 75% occupancy. This value increased to near saturation (>99%) by sampling 3 replicate water samples from 3 locations along a 100m transect. That said, rarefaction curves indicated that additional sampling would have recovered additional species. These results provide important benchmarks for replication and sampling efficiency within nearshore marine environments and highlight the need to adjust sampling intensity and replicates, depending on the questions to be addressed with eDNA.

Despite not achieving saturation with our sampling design, we did observe a transition from exponential to linear addition of species detections with additional sampling similar to that previously demonstrated in mesocosm experiments (Doi et al. 2019). This shift likely reflects the biological reality of eDNA within marine ecosystems, with a few taxa being abundant and a long tail of low abundant species (Kelly et al. 2019). As such, while only a few replicates are needed to capture local core species diversity, high technical (PCR) and biological (bottle) replication is required to saturate species detection (Doi et al. 2019). Thus, if the goal is to detect rare species, it is imperative to increase sampling, an unsurprising result given the reality of detection probabilities of rare taxa (Hunter et al. 2015, Chambert et al. 2015, 2018). Despite this caveat

and our relatively limited number of sample replicates, we still detected rare species such as Giant black seabass (*Stereolepis gigas*) suggesting that eDNA is likely still superior to visual techniques at rare species detection (Thomsen et al. 2012, 2016).

### *Importance of Site Occupancy Modelling*

Site occupancy modeling showed that almost all species (48/50) with occupancy rates higher than 75% were common Southern California kelp forest species with the exception of the Spotted cusk eel and Dog-faced with eel which are both deep-sea species (Love and Passarelli 2020). In contrast, almost all pelagic and intertidal species that should not be present in a kelp forest had low occupancy rates and were detected only in a single bottle replicate (Supplemental Tables 2-S1 & 2-S2). These low occupancy detections cannot be contamination because they did not occur in field or laboratory controls; instead, they likely represent eDNA transported between habitats (Andruszkiewicz et al. 2019). Regardless, site occupancy modeling removed the vast majority of unexpected kelp forest fishes, highlighting its value for determining true species detections in a rigorous and repeatable way (Chambert et al. 2018, Doi et al. 2019), aiding in the interpretation and comparison of eDNA results.

While site occupancy modelling removed non-kelp forest taxa (e.g. Blue whale; *Balaenoptera musculus*; California sea lion, *Zalophus californianus*; pelagic cormorant *Urile pelagicus*; Supplemental Tables 2-S10), it also removed some kelp forest species (e.g. Zebra goby, *L. dalli*; Swell shark, *Cephaloscyllium ventriosum*; Zebra-perch *Hermosilla azurea*; California angel shark, *Squatina californica*,. These results highlight the need for increased replication depending on the management question, just as it may require more visual surveys to observe numerically rarer taxa, such as sharks. Although the ability of eDNA to detect marine mammals and birds is useful, visual observations maybe more effective depending on the taxa,

suggesting that complementary methods may yield the most cost effective sampling regime (Kelly et al. 2017).

### *Diversity inside and outside MPAs*

Traditional visual surveys most often report higher biodiversity and biomass inside MPAs (Ojeda-Martínez et al. 2007, Lester and Halpern 2008, Fox et al. 2014), including Scorpion State Marine Reserve (Pondella II et al. 2015). However, our results surprisingly indicate lower diversity inside the MPA. This paradoxical result is partially explained by the inability of eDNA to resolve *Sebastes* species that were visually observed inside (n=3) and on the edge of the MPA (n=1), but not outside. In addition, despite standardize sample concentration during pooling, sites outside the MPA had ~50% more read depth. Increased read depth should increase species detection, although species rarefaction curves suggest that all samples had sufficient read depth to saturate species richness following site occupancy modelling (Appendix B – Supplemental Figure 2-1).

Instead, a more likely explanation for this unexpected result is that low density of kelp forest fishes outside the MPA increased the detection of non-kelp forest taxa advected from elsewhere. In total, 46.4% of taxa detected outside the MPA were non-rocky reef species such as California angel shark (*Squatina californica*), Chub mackerel (*Scomber japonicus*), and Ocean sunfish (*Mola mola*). Although these species occasionally pass through nearshore rocky reef environments, a more likely explanation is that eDNA from these species were transported from nearby pelagic, intertidal, and sandy bottom ecosystems (O'Donnell et al. 2017, Andruszkiewicz et al. 2019). While such transport would be expected at all sites, high fish abundance inside the MPA would likely result in a strongly skewed ratio of kelp forest eDNA to pelagic eDNA (Hogg et al. 2018), with the signal of kelp forest taxa dominating that of pelagic species.

This paradoxical pattern of species richness highlights that eDNA data must be interpreted with caution (Kelly et al. 2016). Metabarcoding methods often perform unexpectedly when DNA concentrations are low, increasing the probability of sequencing rare species (Goldberg et al. 2016, Deiner et al. 2017). Thus additional ecological metrics to species richness, ones that are more representative of ecological patterns and processes, are needed to optimally interpret eDNA results (McMurdie and Holmes 2013, Chambert et al. 2018). These results ultimately highlight the value of ground truthing eDNA results with visual surveys in novel applications to ensure proper interpretation of results (Kelly et al. 2017).

## **Conclusion**

Marine protected areas are indispensable tools for protecting marine ecosystems. As marine ecosystems continue to decline worldwide due to over harvesting (Bolster, Jeff et al. 2012), ocean acidification (Kroeker et al. 2010), climate change (Filbee-Dexter et al. 2016), coastal eutrophication (Tegner et al. 1995), and the expansion of ocean dead zones (Diaz and Rosenberg 2008), among others, effective MPA monitoring is paramount. Our results demonstrate that eDNA can distinguish fish assemblages inside and outside MPAs, and can detect other vertebrates, like marine mammals and birds, of special conservation concern.

Given its power, ease of sampling and relative affordability, eDNA could provide critical cost-added-benefits of repeated temporal or expanded spatial sampling of marine protected areas. In particular, eDNA metabarcoding can overcome many of the current limitations of visual monitoring, increasing sampling frequency and expanding monitoring beyond a small subset of “important” focal taxa. Such expanded monitoring would improve our ability to understand the ecological processes, human impacts, and management strategies that affect marine community communities that MPAs are designed to protect.

There are important aspects of eDNA that remain to be resolved, most notably determining abundance and biomass via eDNA (Lacoursière-Roussel et al. 2016, Shelton et al. 2019). Until such time, eDNA will likely not be viewed yet as a wholesale replacement for visual monitoring, but instead as a powerful complementary tool. There will always be value in the direct observation by divers, but eDNA provides an important way to simultaneously make surveys more comprehensive and efficient. By replacing aspects of underwater visual surveys, eDNA could reduce the dive time per site, allowing more sites to be surveyed more frequently. Additionally, whereas it takes 3 months for the National Park Service Kelp Forest Monitoring program to complete diver-based surveys, field collection of eDNA could be completed in a week, allowing for surveys to occur during short periods of good weather in the winter when full visual surveys would be impossible. As such, eDNA could greatly expand current monitoring activities across space, time, and depth, providing resource managers critical information on the response of MPAs to changing environments and management practices, and contributing greatly to marine sustainability.



## CHAPTER 3

### **eDNA and Visual Survey Methods Provide Overlapping Yet Divergent Perspectives on Kelp Forest Ecosystems Across the Southern California MPA Network**

#### **Abstract**

eDNA metabarcoding promises a cost effective, automatable, marine biodiversity assessment tool to enhance our ability to characterize marine ecosystems. Here we compare eDNA metabarcoding approaches to two underwater fish census programs, the Channel Islands National Park Service Kelp Forest Monitoring Program and Reef Check California. eDNA and Visual surveys were conducted at 44 sites across the Southern California Marine Protected Area Network to understand the ability of these methods to describe nearshore kelp forest and rocky reef habitats. eDNA methods detected 183 vertebrate taxa including 158 vertebrate species from 4 vertebrate classes compared to the 58 vertebrate taxa including 51 fish species from 2 classes found in Kelp Forest Monitoring program visual surveys. Across all species, eDNA had higher sensitivity (proportion of true positive detections correctly identified as positive) than visual survey methods. However, eDNA failed to consistently identify *Sebastes* and Embiotocidae species due to limitations of the MiFish *12S* barcode for these recent adaptive radiations. Specificity (proportion of true negative detections correctly identified as negative) of Kelp Forest Monitoring Program surveys was significantly higher than eDNA methods, although we observed no difference in specificity between eDNA and Reef Check volunteer surveys. Abundance estimates of both eDNA and visual surveys had markedly high within-site variability making comparisons difficult.

Although we found a few modest relationships between eDNA index abundance scores and biomass (g) and counts for a few species, these relationships were highly variable across all species and visual abundance estimates used. Comparing fish community assemblages across sites, eDNA methods were able to resolve significant differences across the Southern California Bight biogeographic region as well as inside and outside of MPAs. These patterns directly correspond to both concurrent and previous visual observations of marine fish communities along the network. Thus, we demonstrate that eDNA provides ecologically relevant and overlapping, though divergent, estimates of marine fish communities across the Southern California MPA Network. We argue that eDNA methods can provide cost effective scalable assessments of marine biodiversity including the monitoring of the California MPA Network.

## **Introduction**

A key objective of ecological studies is to understand the patterns and mechanisms of the maintenance of biodiversity (Vellend 2010). Fundamental to this goal is the accurate description and characterization of ecosystems, populations, and individuals which interact in complex and often non-linear ways to both abiotic and biotic factors (Dayton et al. 1998, Wells et al. 2017, Caselle et al. 2018, Santora et al. 2020). However, despite technological developments that have revolutionized our ability to characterize many critical aspects of ecosystems through both remote sensing (e.g. kelp forest canopy from LandSat imagery (Hamilton et al. 2020)) and *in situ* deployable monitoring devices (e.g. environmental sample processor (Good et al. 2018)), our ability to accurately and effectively characterize communities remains a persistent challenge (Lessios 1996, Royle and Link 2006, Edgar et al. 2007, Ausubel et al. 2010, Ransome et al. 2017).

This challenge is magnified in marine systems where remote sensing efforts are limited to surface waters and direct underwater observation is severely limited by economic and logistical

constraints (Clauss et al. 2002, Ausubel et al. 2010, Kelly et al. 2014b, Muller-Karger et al. 2018). Importantly, this problem isn't unique to the deep sea where the lack of exploration is a truism (Danovaro et al. 2010), but extends also to more accessible coastal nearshore waters (Edgar et al. 2004, Harvey et al. 2004, Gillett et al. 2012).

Current methods for observing nearshore marine communities rely heavily on visual SCUBA surveys (Hodgson et al. 2004, Sprague et al. 2013). Although a diversity of methods exist, they largely rely on divers to identify and count marine species and are thus affected by observer biases (Edgar et al. 2004). In the case of visual fish census methods, observer bias can result from a variety of factors including species response to the presence of divers (Lindfield et al. 2014), habitat complexity (Green et al. 2013), taxonomic expertise (Edgar et al. 2004), dive conditions (Bozec et al. 2011), and mismatch between species activity and the time surveys are conducted (Bassett and Montgomery 2011). In particular, visual fish surveys often fail to capture transient and migratory predators that rarely utilize one particular reef, but can play an outsized role in ecosystem structuring (Lowe et al. 2006, Thomsen et al. 2016, Bakker et al. 2017, Lafferty et al. 2018). Also difficult to monitor are cryptic benthic species that require careful and time consuming observation in and around crevices and macroalgae (Willis 2001).

In addition to the above, worker safety constraints limit the length and depths of dive surveys, typically to 1-2 hours duration and less than 30 m in depth (Edgar et al. 2004). Even with such a limited scope, visual scuba surveys can still be expensive, and often can require large and specialized vessels and crews of trained divers (Hodgson et al. 2004, Sprague et al. 2013). Combined, these issues largely limit monitoring efforts to economically important and conspicuous fish species, while restricting surveying efforts to only a handful of sites, typically once per year (Willis 2001, Pondella II et al. 2015).

By limiting the scope and scale of marine ecosystem surveys, the above challenges limit our understanding of their ecological health and function. Given the growing threats to marine ecosystems worldwide (Fabry et al. 2008, Halpern et al. 2008, Diaz and Rosenberg 2008, Bolster, Jeff et al. 2012, Doney et al. 2012), it is essential to develop alternative methods that expand our ability to observe and monitor marine communities. One increasingly popular approach is eDNA metabarcoding (Kelly et al. 2014a, Miya et al. 2015), a method that relies on the capture and sequencing of DNA left behind by resident marine species, allowing for the reconstruction of communities from the collection and processing of sea water (Ficetola et al. 2008). Given the ease of sample collection, declining costs of molecular reagents and sequencing, and the potential for automation, eDNA has the potential to overcome many of the logistical constraints posed by visual underwater census observations (Good et al. 2018, Yamahara et al. 2019), representing a potential alternative to SCUBA-based visual surveys for monitoring and surveying marine communities (Kelly et al. 2014b).

Previous comparisons of eDNA and visual methods reveal overlapping but different views of marine communities (Thomsen et al. 2012, 2016, Kelly et al. 2014a, 2017, Ushio et al. 2018a, Closek et al. 2019). Almost universally, eDNA detects a broader range of taxa than visual surveys (Port et al. 2015, Valentini et al. 2016, Yamamoto et al. 2016, Ushio et al. 2018b), but frequently fails to detect a small subset of species observed visually, resulting in differing community patterns across sites (Kelly et al. 2017). Furthermore, species abundance, not just diversity, is a critical measures of marine ecosystem health (Tegner 2000, Edgar et al. 2007, Lester and Halpern 2008, Gaines et al. 2010), and the utility of eDNA to estimate abundance remains equivocal (Iversen et al. 2015, Evans et al. 2016, Klobucar et al. 2017, Fonseca 2018, Rice et al. 2018, Chambert et al. 2018, Tillotson et al. 2018, Pont et al. 2018). Despite its promise, eDNA research is still in its

infancy. Few studies have directly compared eDNA and visual estimates of marine communities to test whether they yield produce similar ecological patterns (Kelly et al. 2014b, Bohmann et al. 2014), information that is critical to inform future applications of eDNA metabarcoding approaches for monitoring marine ecosystems.

One key application of marine ecosystem monitoring is marine protected area (MPA) management (Pondella II et al. 2015). MPAs are important conservation tools for preserving and enhancing resource stocks, maintaining ecosystem function, and promoting ecosystem resilience (Edgar et al. 2007). Effective MPA monitoring is critical to ensure MPAs are producing intended benefits (Carr et al. 2011), but current MPA monitoring efforts largely rely on visual survey methods (Hodgson et al. 2004, Sprague et al. 2013). This is true in the Southern California MPA Network, which was established in 2012, covering 16% of nearshore waters along 544 km of coastline and 6 Channel Islands (Costa et al. 2013, Gleason et al. 2013, Saarman et al. 2013).

The Southern California MPA Network is monitored collaboratively by academic, non-profit, state and federal agencies that survey 94 kelp forest and rocky reef sites, but typically just once per year (Pondella II et al. 2015). These surveys focus on key indicator taxa including macroalgae, benthic invertebrates, and fishes. Results indicate these MPAs are successful, with higher biomass of target species within reserves, particularly fishes (Claisse et al. 2013). In addition, these surveys reveal a strong biogeographic pattern of kelp forest fish communities that span a sea surface temperature gradient driven largely by the confluence of the California Current and California Counter Current (Hamilton et al. 2010, Claisse et al. 2018).

Because the Southern California kelp forest ecosystem is one of the most surveyed marine ecosystems globally (Pondella II et al. 2015) with well-known differences in fish community assemblages (Hamilton et al. 2010, Claisse et al. 2018), it is an ideal system to rigorously compare

eDNA and visual underwater census surveys. To better understand how these methods, compare and to test the utility of eDNA for MPA monitoring, this study compares eDNA metabarcoding and visual fish surveys broadly across the Southern California MPA network.

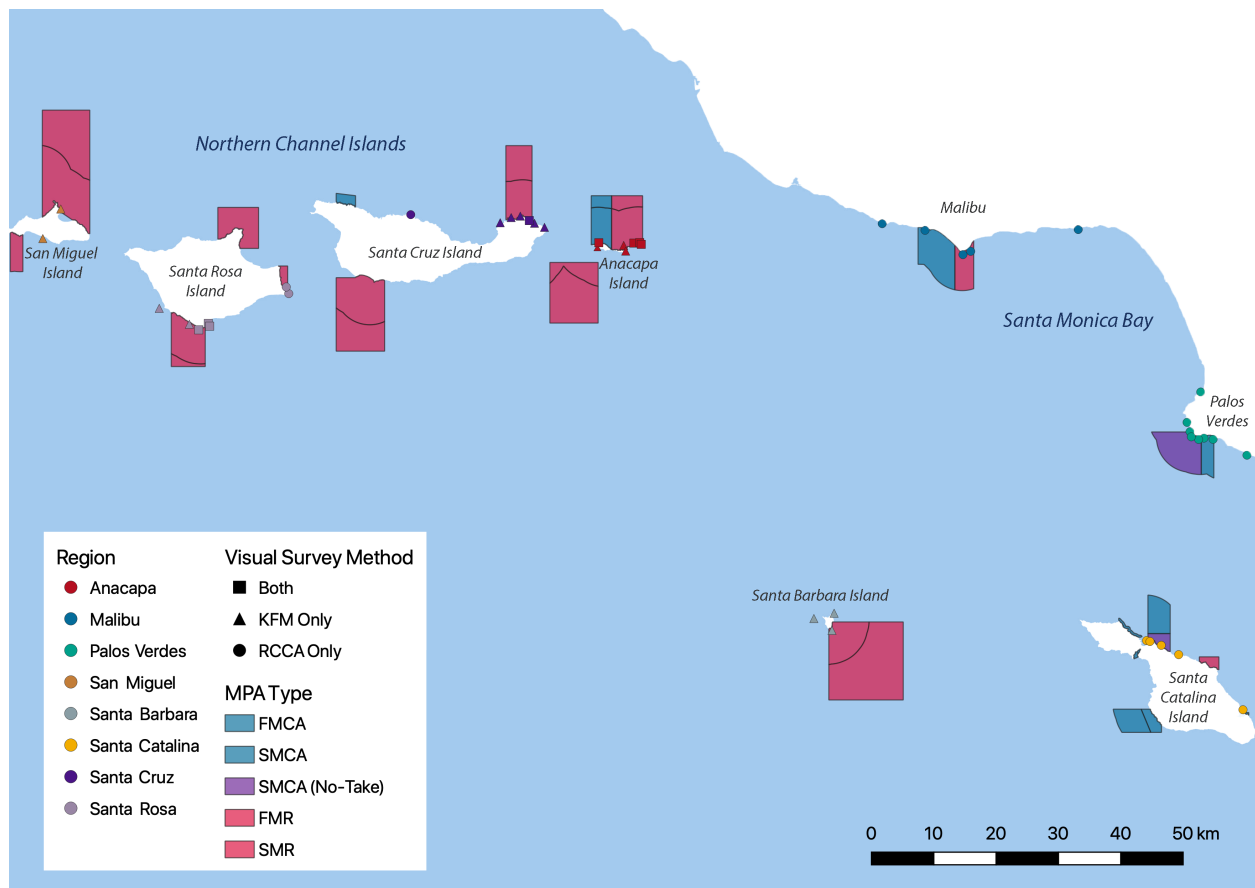
## **Materials and Methods**

### *Sites Samples*

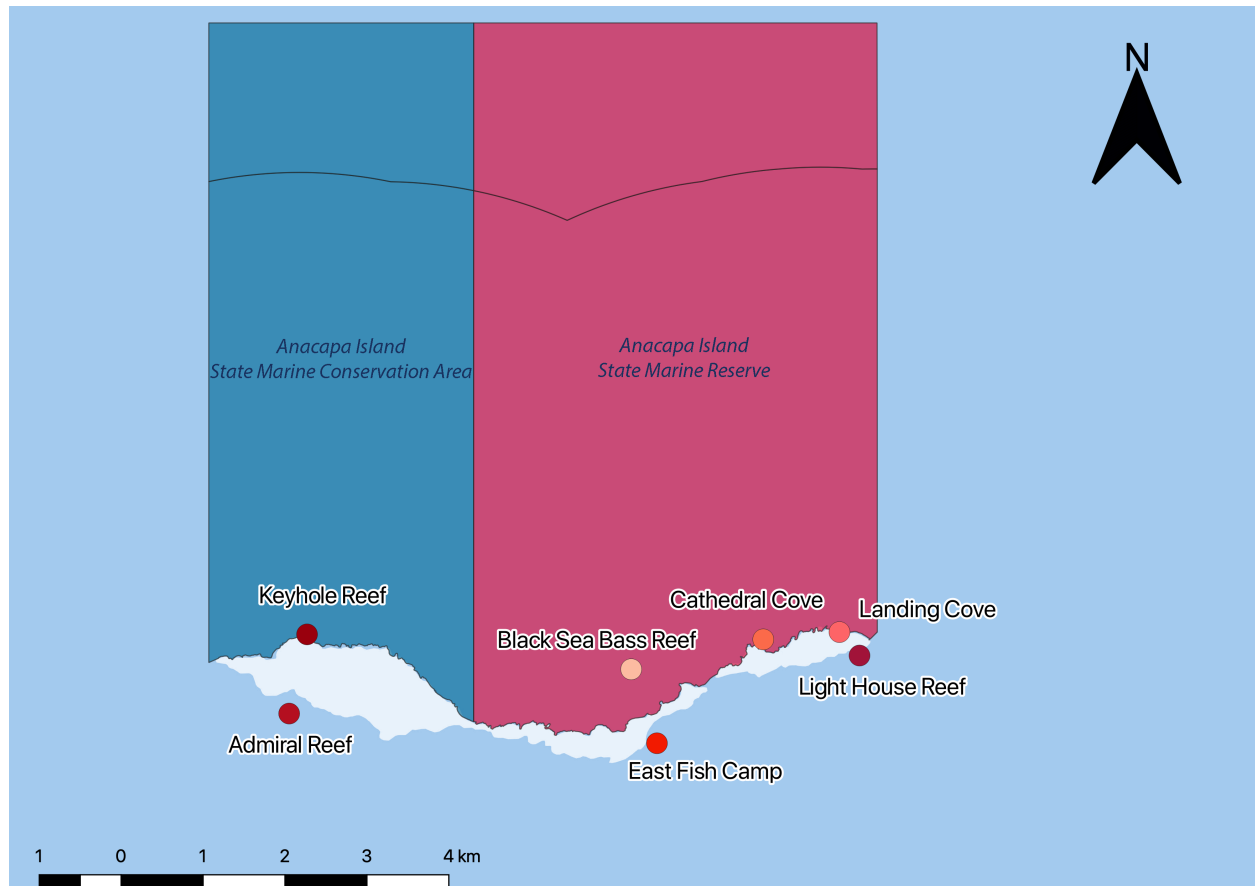
We conducted eDNA and visual surveys at 44 sites in the summer of 2017, sampling both inside and outside of protected habitats (Figures 3-1 through 3-9). All sites were previously chosen as part of long term kelp forest and nearshore rocky reef monitoring programs (Gillett et al. 2012). Of these sites, 16 are monitored by the Channel Islands National Park Service Kelp Forest Monitoring Program (hereafter Kelp Forest Monitoring Program), 20 are monitored by the community science non-profit Reef Check California (hereafter Reef Check), and 8 sites are monitored by both programs. Of these 44 sites, 22 are located within 12 distinct MPAs including two State Marine Conservation Areas that allow limited recreational take, 3 within no-take State Marine Conservation Areas, and 7 within no-take State Marine Reserves.

At four of the above MPAs, we surveyed at least two monitoring sites within the MPA and two unprotected reference sites outside of the MPA (Supplemental Table 3-S1). For the remaining 11 MPAs, we sampled at least one site within the MPA and one paired reference site outside the MPA (Supplemental Table 3-S1). These sites are distributed across 6 Channel Islands (Catalina, Santa Barbara, Anacapa, Santa Cruz, Santa Rosa, and San Miguel Islands) and 2 unique mainland headlands (Malibu and Palos Verdes) spanning the confluence of the California Current and California Counter Currents.

**Figure 3-1. Site Map.** Sites are color coded by region and shapes depict which visual survey program monitored that site. Marine protected areas are shaded by type and outlined. Partial take State Marine Conservation Areas (SMCA) and Federal Marine Conservation Areas (FMCA) are shaded dark blue. No-take SMCA's are shaded purple. No-take State Marine Reserves (SMRs) and Federal Marine Reserves (FMRs) are shaded in red.

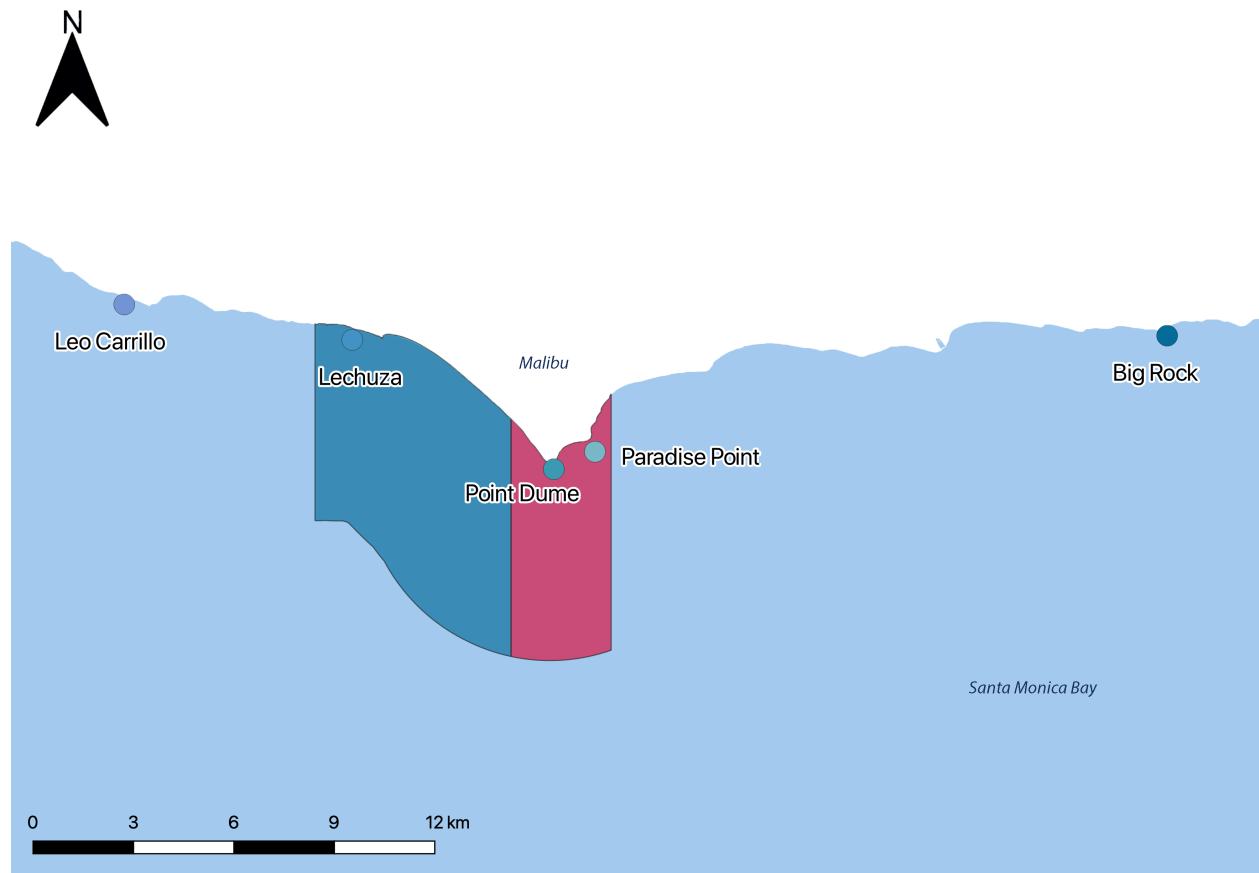


**Figure 3-2.** Anacapa Island Site Map

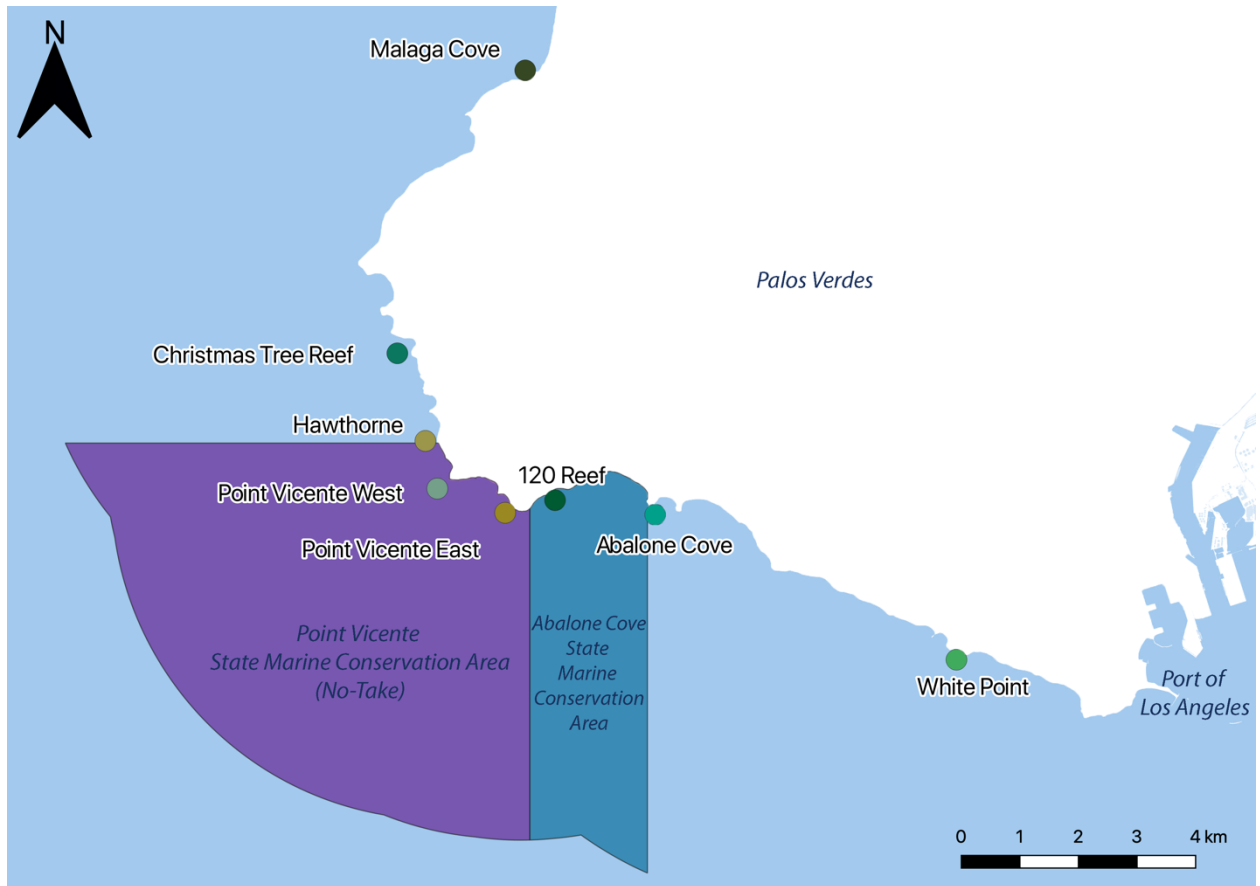




**Figure 3-3. Malibu Site Map**



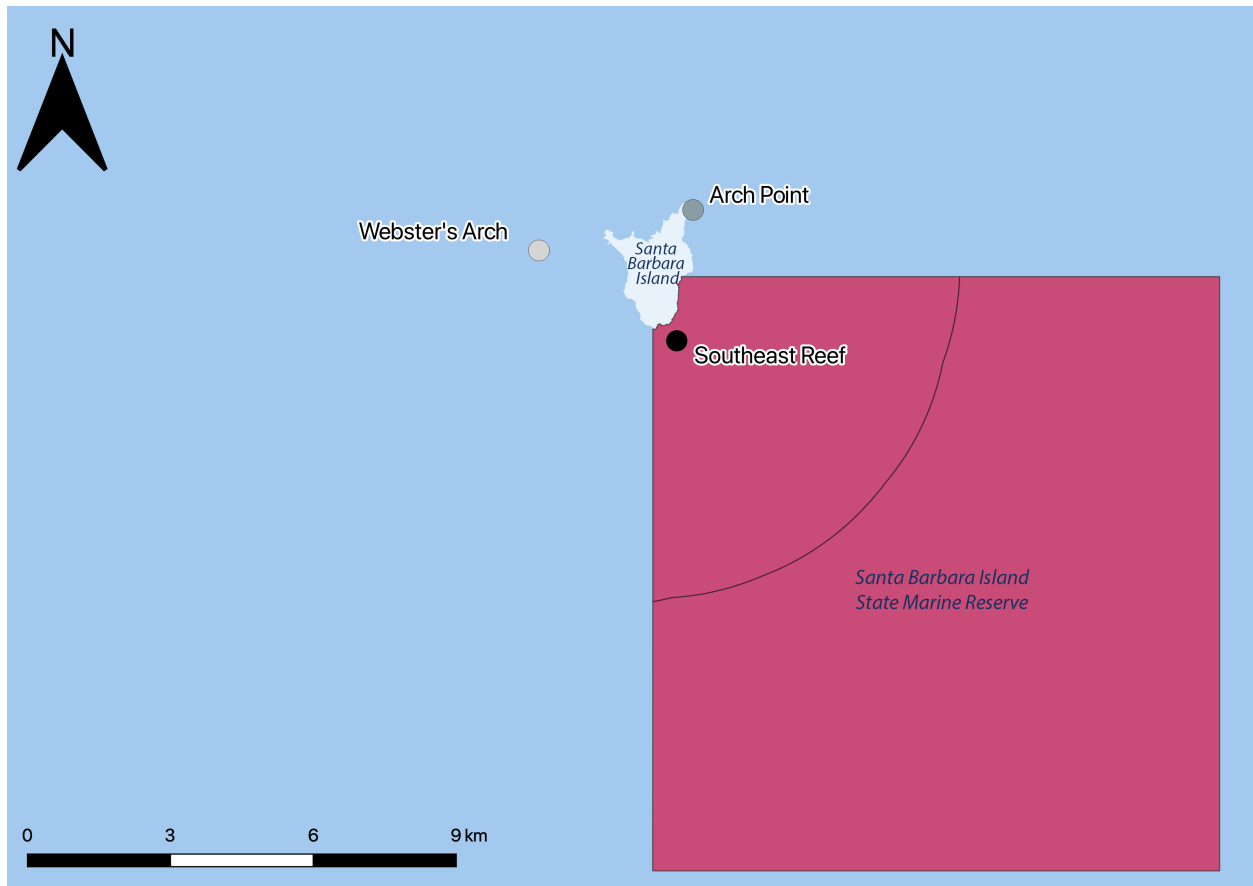
**Figure 3-4. Palos Verdes Site Map**



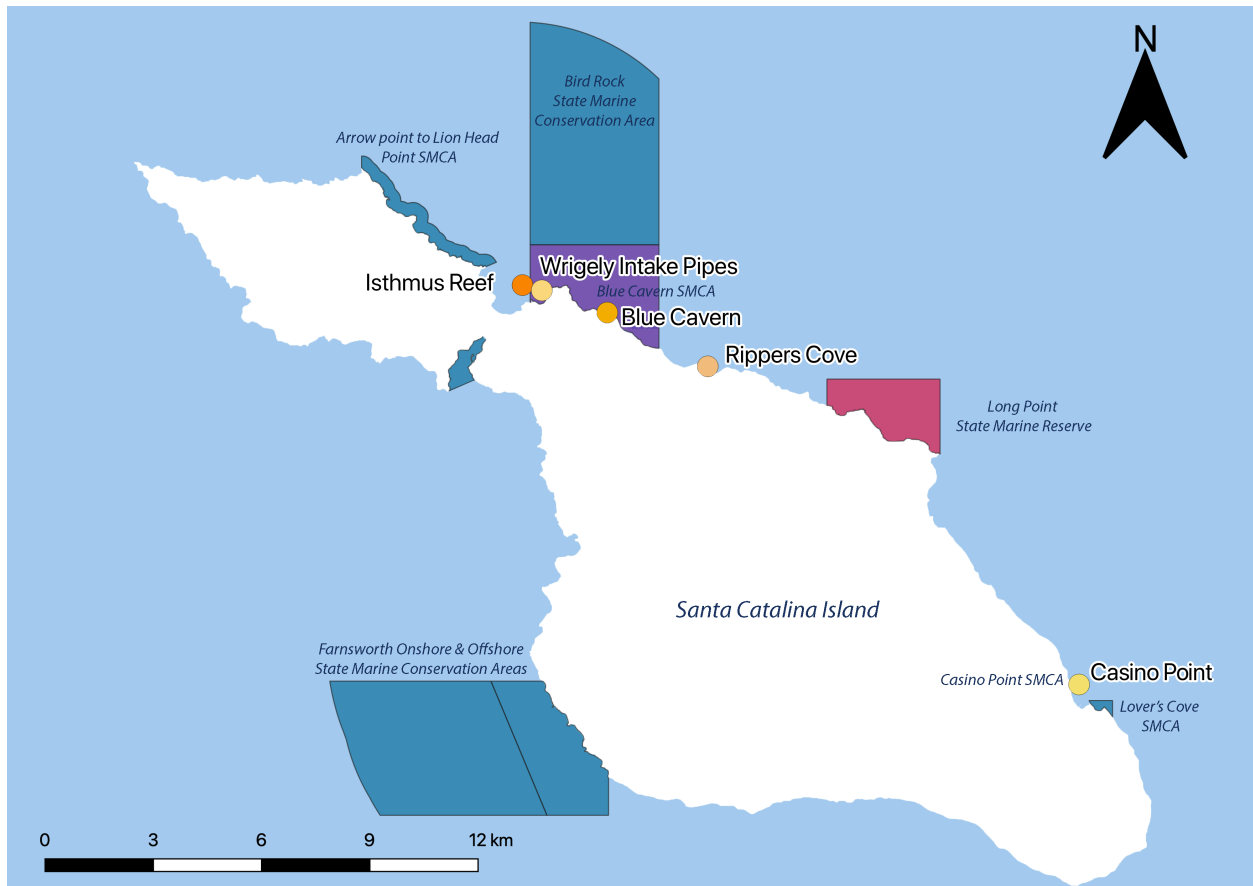
**Figure 3-5.** San Miguel Island Site Map



**Figure 3-6.** Santa Barbara Island Site Map



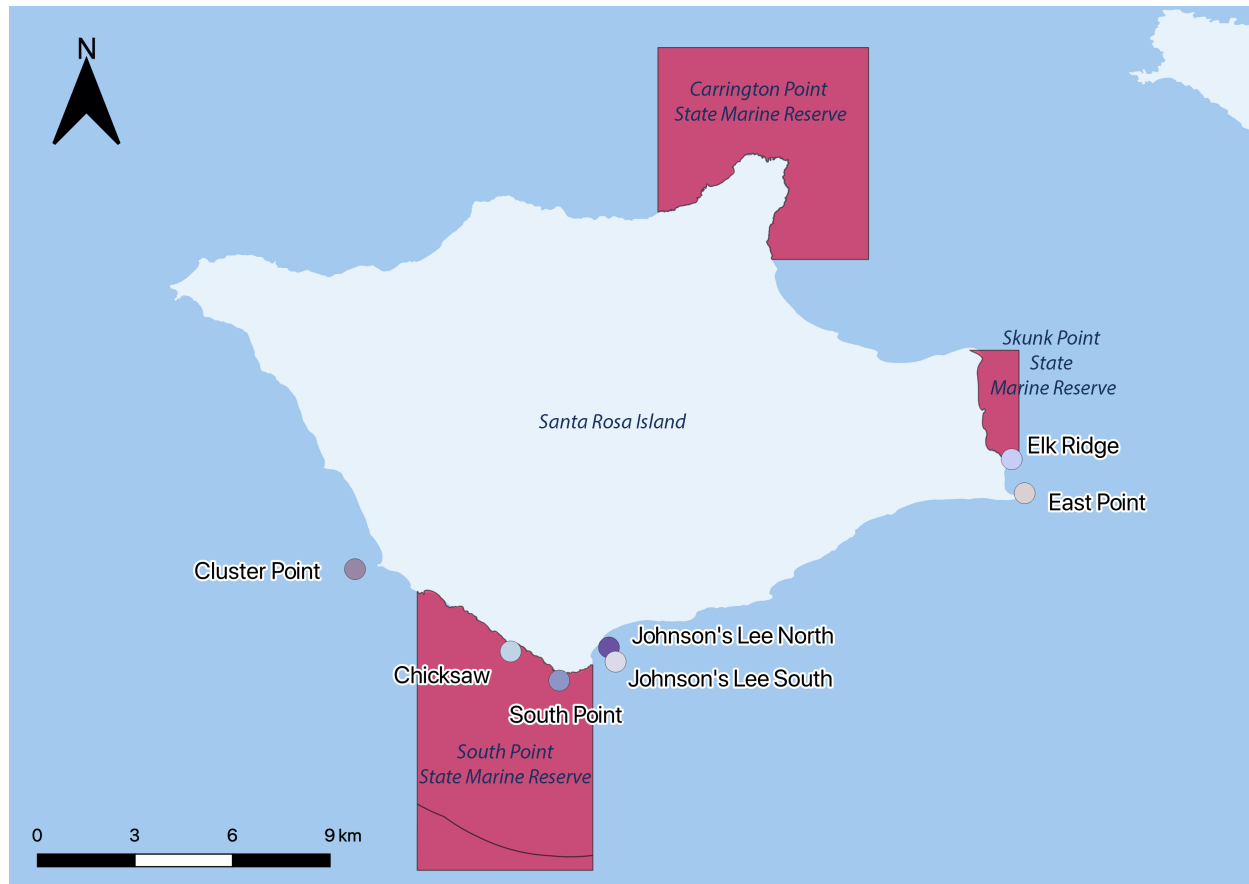
**Figure 3-7. Santa Catalina Island Site Map**



**Figure 3-8.** Santa Cruz Island Site Map



**Figure 3-9.** Santa Rosa Island Site Map



*Kelp Forest Monitoring Program Visual Underwater Census*

The Kelp Forest Monitoring Program surveys were conducted between May 8<sup>th</sup>, 2017 and September 27<sup>th</sup>, 2017 (Supplemental Table 3-S1). The Kelp Forest Monitoring Program conducted visual fish surveys at 24 established monitoring sites around the Northern Channel Islands following standard protocols (Graham 2004, Halpern et al. 2006, Gillett et al. 2012, Kushner et al. 2013, Sprague et al. 2013). Briefly, fish species are recorded along a permanent 100 m transect at each site, employing visual fish transects, 1m quadrats, fish size-frequency surveys, and roving diver fish counts. Visual fish transects are conducted to specifically determine the abundance of 13

indicator species at each site. They are conducted by having one diver record midwater taxa and a second record benthic taxa within a 3 m tall x 2 m wide x 50 m long area. Fish size-frequency surveys are conducted to determine fish population size structure of all observed fish species at each site. Fish size-frequency surveys are conducted by having at least one diver slowly swim back and forth within 10 m of either side of the 100 m transect line for 30 minutes counting and recording to length (to nearest cm for fish <15 cm total length and nearest 5 cm for larger fish) for all fish species observed. Roving diver fish counts are conducted to estimate species diversity and abundance of all fishes within the entire transect area. To conduct roving diver fish counts, two divers slowly swim a 100 m transect line for 30 minutes estimating fish abundance by both direct counts and on a non-linear 0-10 scale for species observed within 10m of either side of the transect line. Two estimates of abundance are used to both allow for direct historical comparisons of methods as well as to account for difficulties in counting hundreds to thousands of individuals on a single transect. For both fish size frequency and roving diver fish counts surveys, any fish species observed is counted; over the last 40 years, a total of 178 species of fish have been observed (See Chapter 2 methods). Lastly, the 1m<sup>2</sup> quadrat surveys are conducted to count the abundance of 3 small benthic species. Twelve paired 1m<sup>2</sup> quadrat surveys (n=24) are systematically placed 8.33m apart from each other with a random starting location chosen each field season.

### *Reef Check California Visual Underwater Census Methods*

Reef Check surveys were conducted between June 17<sup>th</sup>, 2017 and November 22<sup>nd</sup>, 2017 (Supplemental Table 3-S1). Trained Reef Check volunteers conducted visual fish transects at 28 sites between Palos Verdes and the Northern Channel Islands (Hodgson et al. 2004, Gillett et al. 2012). Briefly, at each site divers record number and estimate size (to nearest cm) of 35 indicator species within a 2 m x 2 m window along a 30 m transect. We note that unlike the Kelp Forest



Monitoring program, Reef Check surveys are designed to only monitor this specific list of marine fish taxa and do not record observations of other taxa even if present. At each site 18 transects are deployed in three perpendicular-to-shore columns of 6 parallel-to-shore rows. Exact location of each transect deployment is haphazardly chosen by the dive team leader. However, each row of 6 transects is roughly at the same depth, all transects are at least 5 m apart from any other transect, and all transects are oriented along the same compass heading (Hodgson et al. 2004).

#### *Visual Data Biomass Calculations and Data Transformation*

Total number of species were calculated from combining the each of the four distinct types of Kelp Forest Monitoring surveys. However, for comparisons across the biogeographic area and inside and outside MPAs, data from each of the four distinct types of Kelp Forest Monitoring Program surveys were analyzed individually. For visual fish transects, the individuals per transect were used. For 1m quadrats, the individual per quadrat were used. For fish size-frequency surveys, biomass (g) for each species was estimated using previously published length-weight relationships (Williams et al. 2013) (Supplemental Table 3-S2). For roving diver fish count surveys, data were analyzed using both individuals per transect as well as the relative abundance score described above. Data from the Reef Check surveys were analyzed both using counts of individuals per transect as well as biomass (g) estimated using previously published length-weight relationships (Supplemental Table 3-S3).

We created species-by-site community tables for each survey method in two separate ways: 1) across all species and each individual transect, and 2) site averaged data table across all species. We used the species-by-site community tables to investigate both the relative variance explained by transect replication as well as sub-region MPA comparisons. Lastly, to investigate patterns

across the MPA Network and biogeographic region we used the site averaged species-by-site community table.

### *eDNA Sample Collection*

We collected eDNA samples between May 10<sup>th</sup>, 2017 and September 18<sup>th</sup>, 2017. We conducted sampling along the same fixed transects used by the Kelp Forest Monitoring Program within 95 days of visual surveys (mean = 26.8 days). For Reef Check sites, we collected eDNA samples along the “core” transect nearest to 10 m depth within 80 days of visual surveys (mean = 25.5 days; Supplemental Table 3-S1).

At each site, we collected three replicate 1 L water samples (“biological replicates”) from 10 m below the surface (or deepest site depth) and 1 m above the benthos using either a 4 L Niskin bottle deployed from the UCLA RV Kodiak or on SCUBA (Supplemental Table 3-S1) (Port et al. 2015, Thomsen et al. 2016). At four sites sampled by both Reef Check and the Kelp Forest Monitoring Program on separate days, we collected 2 sets of samples with each separate visual survey method (Supplemental Table 3-S1). From each Niskin deployment, we transferred a single liter of seawater to an enteral feeding pouch, similar to an intravenous (IV) bag (hereafter “pouch”), which we then hung to facilitate gravity filtration of eDNA through a sterile 0.22 µm Sterivex (Millipore Sigma, Burlington, MA, USA) cartridge filter in the field (Port et al. 2015, Miya et al. 2015, Curd et al. 2019). For samples collected via SCUBA, we filled 1 L pouches with seawater at 10 m depth, 1 m above the bottom, and then gravity filtered the samples at the conclusion of the dive as described above. Finally, we dried these filters, using a 3 mL syringe to force out any remaining seawater, and then capped and stored the filters at -20°C for DNA laboratory work at UCLA (Miya et al. 2015).

To minimize carryover between sites, we thoroughly rinsed the Niskin bottle at the surface using seawater. To minimize carryover between replicate samples at a single site, we left the Niskin bottle open at the surface to flush with seawater for 30 seconds. Further, the Niskin was also left open during each cast, allowing the 10 m water column to pass through the bottle, further diluting any carryover from previous casts. To control for potential contamination, we processed 18 field blanks as a negative control, one per sampling excursion immediately after a day of sample collection. Prior to taking the field blank, we rinsed the recently deployed Niskin with freshwater for 30 seconds, and then filled the Niskin with 1 L of distilled water (Goldberg et al. 2016, Thomsen et al. 2016). This water was then transferred to a pouch for filtering as described above.

#### *DNA Extraction and Library Preparation*

We extracted eDNA from the Sterivex cartridge using a modified DNAeasy Blood and Tissue Kit protocol (Spens et al. 2017) (Qiagen Inc., Germantown, MD), directly adding proteinase K and ATL buffer inside the filter cartridge before an overnight incubation. We then PCR amplified the extracted eDNA using both the MiFish Universal Teleost and MiFish Universal Elasmobranch *12S* primer sets (Miya et al. 2015) with Nextera modifications following the methods of Curd *et al.* (Curd et al. 2019). For each 1 L replicate, we conducted three technical PCR replicates in triplicate for each barcode (9 PCRs per site per barcode), which were each treated as a unique sample throughout the library preparation process. We performed PCR amplification for both primer sets in a 25  $\mu$ L reaction volume containing 12.5  $\mu$ L Multiplex Taq PCR 2x Master Mix (Qiagen Inc., Valencia, CA, USA), 6.5  $\mu$ L of dH<sub>2</sub>O, 2.5  $\mu$ L of each primer (2  $\mu$ mol/L), and 1  $\mu$ L DNA extraction. PCR thermocycling employed a touchdown profile with an initial denaturation at 95°C for 15 min to activate the DNA polymerase followed by 13 cycles

of a denaturation step at 94°C for 30 sec, an annealing step starting at 69.5°C for 30 sec which was then decreased by 1.5°C for each cycle (last cycle was 50°C), and an extension step at 72°C for 1 min. This initial touchdown was followed by 35 additional cycles carried out at an annealing temperature of 50°C using the same denaturation and extension steps above, and ending with a final extension at 72°C for 10 min (Curd et al. 2019). All PCRs included a negative control where molecular grade water replaced the DNA extraction. For a positive control, we used DNA extractions of American Alligator (*Alligator mississippiensis*) and Dromedary (*Camelus dromedarius*) both terrestrial species non-native to California. To ensure amplification success and correct product size, we electrophoresed all PCR products on 2% agarose stained with SybrGreen.

We prepared each unique technical PCR replicate generated from sea water samples as a separate metabarcode library. We pooled 5 µL of all negative PCR controls (all without bands) into a single pooled negative PCR control. Only the single pooled negative PCR control and two positive controls were included in the library preparation and sequencing runs. We then cleaned all pooled samples using Serapure magnetic beads (Faircloth and Glenn 2014) and quantified their concentrations using the high sensitivity Quant-iT™ dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) on a Victor3 plate reader (Perkin Elmer Waltham, MA, USA) and indexed our sequencing libraries using the Nextera Unique Dual Index A, B, C and D Kits (Illumina, San Diego, CA, USA) following the methods of Curd et al. (2019). The indexing PCR was performed using a 25 µL reaction mixture containing 12.5 µL of Kapa HiFi HotStart Ready mix (Kapa Biosystems, Wilmington, MA, USA), 0.625 µL of primer i7, 0.625 µL of primer i5, and 10 ng of template DNA, and used the following thermocycling parameters: denaturation at 95°C for 5 min, 5 cycles of denaturation at 98°C for 20 sec, annealing at 56°C for 30 sec, extension at 72°C for 3 min, followed by a final extension at 72°C for 5 min (Curd et al. 2019). We electrophoresed

all indexed PCR products on a 2% agarose gels to confirm successful PCR and correct product size. Following bead cleaning and quantification, as described above, we pooled the indexed libraries in equimolar concentration by barcode and technical replicate (e.g. MiFish Teleost Technical Replicate 1 across all samples). We then sequenced each of the 6 resulting libraries on a NextSeq PE 2x150bp mid-output at the Technology Center for Genomics & Bioinformatics (University of California- Los Angeles, CA, USA), using 20% PhiX added to all sequencing runs.

### *Bioinformatics*

To determine community composition from metabarcode sequences, we used the *Anacapa Toolkit*, which conducts quality control, amplicon sequence variant (ASV) parsing, and taxonomic assignment using user generated custom reference databases (Curd et al. 2019). Briefly, we processed each library separately using the default parameters. Taxonomic assignments were conducted twice for all libraries: first using a complete *CRUX*-generated *I2S* reference database using all publicly available reference sequences and second using the curated California fish specific reference database FishCARD (Gold *et al.* in prep). We then selected all mammal and avian taxonomic assignments obtained using the *CRUX*-generated *I2S* reference database and replaced the respective empty taxonomic assignments obtained using the FishCARD database. We then transferred the resulting species community tables into *R* for subsequent downstream data analysis (Team 2014).

### *Decontamination and eDNA Data Transformation*

Prior to analyses, we decontaminated the raw ASV (unique sequence derived from metabarcoding) species-by-sample community table of each library separately to ensure that potential field contamination, lab contamination, and sequence index hopping could not influence

the results (Goldberg et al. 2016, Costello et al. 2018). Following Kelly et al. (2018) and McKnight et al. (2019), we implemented a three step decontamination protocol: the first identified and removed sequences arising from index hopping, the second identified and removed sequences from negative controls, and the third conducted site occupancy modeling to identify true positives detections (Royle and Link 2006, Schmidt et al. 2013, Ficetola et al. 2016, Lahoz-Monfort et al. 2016; See Appendix C – Supplemental Methods).

Next, we transformed the resulting eDNA species-by-sample community tables into eDNA index scores following Kelly et al. (2019) creating three distinct eDNA community tables for subsequent data analysis: 1) eDNA index scores calculated across all technical replicates (herein species-by-technical replicate table), 2) eDNA index scores averaged across biological replicates merging community tables created from both metabarcoding primers (herein species-by-biological replicate table), 3) eDNA index scores calculated per site, averaging across all technical and biological replicates and for both barcodes (herein species-by-site table). The eDNA index score was computed by first calculating the mean read count for each assigned taxonomy and then calculating the percent abundance of each ASV; the number of reads of each ASV divided by the total number of reads per sample (1: technical replicate, 2: biological replicates, 3: site). The relative abundance of each taxon in each sample was then divided by the maximum abundance for a given species across all samples to generate the eDNA index. The index thus normalizes the read count per species and per sample. The eDNA index values 0 to 1 for each taxa, allowing for abundance comparisons of a specific taxa across sites.

The eDNA species-by-technical replicate data table was used to investigate the relative variance explained by technical replication, biological replication, unique metabarcoding primer, and sites sampled. The eDNA species-by-biological replicate averaged data table was used to

investigate sub-region MPA comparisons. The eDNA species-by-site averaged data table was used to investigate patterns across the MPA Network and biogeographic region.

### *Comparison of eDNA and Visual Underwater Census Methods*

We compared community composition detected by eDNA results to Kelp Forest Monitoring Program and Reef Check visual census results separately. First, we identified taxa detected by eDNA and each visual census program, taxa observed in each visual census program but not eDNA, and taxa detected by eDNA but not each visual census program. Second, we calculated site occupancy rates of each visual survey program's distinct survey methods as well as eDNA approaches according to Chambert *et al.* (Chambert et al. 2018). Site-occupancy modeling provides a robust statistical framework to determine if the presence pattern of a given species reflects a PCR artifact or rare organism (Royle and Link 2006, Schmidt-Roach et al. 2013). The binomial model yields the likelihood that a species detected is truly present in the sample. The model, implemented in *Stan for R* (<https://mc-stan.org/>; Team 2014), depends upon three parameters: 1) the commonness of a species in the dataset or occupancy rate (denoted Psi), 2) the probability of a detection given that the species is truly present (true positive detection; denoted P11), and 3) the probability of a detection given that the species was not truly present (false positive; denoted P10). The probability occurrence function used was the following:

$$\text{Probability of Occurrence} = (\text{PSI} \times (\text{P11}^N) \times (1-\text{P11})^{(K-N)}) / ((\text{PSI} \times (\text{P11}^N) \times (1-\text{P11})^{(K-N)}) + ((1-\text{PSI}) \times (\text{P10}^N) \times ((1-\text{P10})^{(K-N)})))$$

Where K is the number of samples taken within a site and N is the number of species detections within a site.

We used sensibly informative priors for parameter estimations. First, we assume that our primers do a reasonably good job of detecting species if the species are present (Chambert et al. 2018, Doi et al. 2019). Thus, true positive probability ( $P_{11}$ ) and occupancy probability ( $\Psi$ ) were modeled with priors from a left-skewed beta distribution where  $\alpha = 2$  and  $\beta = 2$ . We also assumed that the false-positive rate of detection is unlikely to approach the true-positive rate. Thus, false positive probability ( $P_{10}$ ) was modeled with priors from a left-skewed beta distribution where  $\alpha = 1$  and  $\beta = 10$ . *Stan* occupancy models are included in Supplemental Materials as attached text files.

For each species detected by eDNA methods, we calculated the number of detections out of the number of technical PCR replicates and water samples taken at each site. The occurrence of a sequence for one species in a given replicate was treated as a detection at that site. Each pattern of occurrence for a given species within a given water sample was considered a case (e.g. 2 detections out of 3 PCR replicates). We then summarized the number of occurrences of each case and ran each case through a separate occupancy model to reduce computational time.

For each species detected by a particular visual survey method we calculated the number of detections out of the number of transects or quadrats taken at each site for a given species. Rates were calculated as described above.

Each unique model was run at least 10 times with 10,000 iterations in order to filter out cases in which the model converged into a local maximum. Because the Kelp Forest Monitoring program 1 m quadrat surveys had too low of an effective sample size at 10 model runs, we increased the total number of model runs to 25 to ensure convergence.

We then calculated sensitivity and specificity of eDNA and visual surveys for each species detected using the estimated true positive and false positive rates obtained by the site occupancy modeling. The false negative rate is  $1 - \text{true positive rate}$  and the true negative rate is  $1 - \text{false}$



positive rate. Sensitivity was calculated using the following equation: True Positive Rate / (True Positive Rate + False Negative Rate). Specificity was calculated using the following equation: True Negative Rate / (True Negative Rate + False Negative Rate). We then calculated the average sensitivity and specificity for eDNA and each visual survey methods. To compare sensitivity and specificity between eDNA and visual survey methods we conducted student's T-test across the sensitivity and specificity values of all species detected by each method.

To test the ability of eDNA to capture abundance, as well as diversity data, we compared eDNA index scores to abundance data from visual surveys. We calculated abundance from visual census data both in terms of individuals per transect directed from census data and biomass per transect. We then fit linear regressions between average eDNA index scores and average counts and biomass from each pairwise site comparison.

We then compared the variation between levels of replication for eDNA and visual survey methods using a nested PERMANOVA to determine what the fraction of within site variance can be explained by the level of replication employed for both methods. We first calculated pairwise Bray-Curtis dissimilarities between all replicates for each method independently. For eDNA surveys we then fit a hierarchical nested PERMANOVA on the Bray-Curtis dissimilarity matrix using with the following equation:

$$\text{eDNA\_index} \sim \text{Site/Primer} + \text{Site/Primer/Bio\_rep.} + \text{Site/Primer/Bio\_rep./Technical\_rep.} + \text{Site}$$

The nesting structure was as follows: Technical replicates (PCRs) were nested within biological replicates (bottles), biological replicates were in turn nested within primer sets (teleost or elasmobranch), and primer sets were nested within sites. In contrast, visual surveys only had one level of nesting per site: transects were nested within sites. We calculated pairwise Bray-Curtis

dissimilarities using both biomass (g) and counts. The two hierarchical nested PERMANOVA equations were as follows:

- 1) Biomass (g)  $\sim$  Site/Transect + Transect.
- 2) Count  $\sim$  Site/Transect + Transect.

For all survey methods, we report total amount of variation explained by each level of replication for both eDNA and visual transect surveys. Results were then visualized through NMDS ordination.

Finally, we compared geographic variation in community structure across all sites surveyed by eDNA and both visual methods. We first calculated average eDNA index scores at each site as well as average biomass and count at each site for both visual survey methods. We then calculated pairwise Bray-Curtis dissimilarities between sites for each method separately, fitting the following hierarchical nested PERMANOVA to the above dissimilarity matrices to account for the strong biogeographical signal known to occur across the region: Region/MPA + Region. We conducted eDNA region-wide MPA analyses in multiple ways: 1) comparing eDNA data from all sites and all detected species, 2) comparing eDNA data from all sites and only fish species, and 3) comparing eDNA data from all sites but only for species observed by both visual and eDNA. We then repeated the above three analyses of eDNA data focusing on sites only surveyed by each visual survey method respectively. For both visual survey methods, region-wide MPA analyses were conducted using all sites and species collected for each method respectively.

Given the known biogeographic patterns across regions within our study, we then conducted within-region analyses of both the eDNA biological replicate data and visual survey method transect data for each of the 8 regions surveyed to compare fish communities inside and outside MPAs. As above, we calculated within-region pairwise Bray-Curtis dissimilarities between

biological replicates and transects respectively. We then fit the following PERMANOVA to the dissimilarity matrices to test for differences in fish communities inside and outside of MPAs within each region for each of the three methods: MPA + Site.

All significant PERMANOVA results were then followed by *betadisper* test comparing the homogeneity of dispersions across each tested variable (Oksanen et al. 2013). All PERMANOVA and *betadisper* tests were run with 10,000 permutations.

## Results

### *eDNA Results*

We generated over 290 million reads that passed Illumina NextSeq quality control filters, 188 million MiFish Teleost reads and 102 million MiFish Elas reads. Combined, the *Anacapa Toolkit* identified 7,854 ASVs from 93.2 million reads representing 918 samples, 71 ASVs from 156,694 reads representing 52 blanks, and 82 ASVs for 5,789,050 for 12 positive controls. After decontamination we retained 6,580 ASVs from 76.7 million reads representing 778 samples (Supplemental Table 3-S1), resulting in the detection of 135 vertebrate taxa, representing 108 unique species, 98 genera, 57 families, and 4 classes (Supplemental Table 3-S4). The strict decontamination methods employed here resulted in the exclusion of 140 technical replicates; remaining samples had a median of 8 MiFish Elas technical replicates and 9 MiFish Teleost technical replicates (max = 18 and min = 3) (Supplemental Table 3-S1).

### *Visual Survey Results*

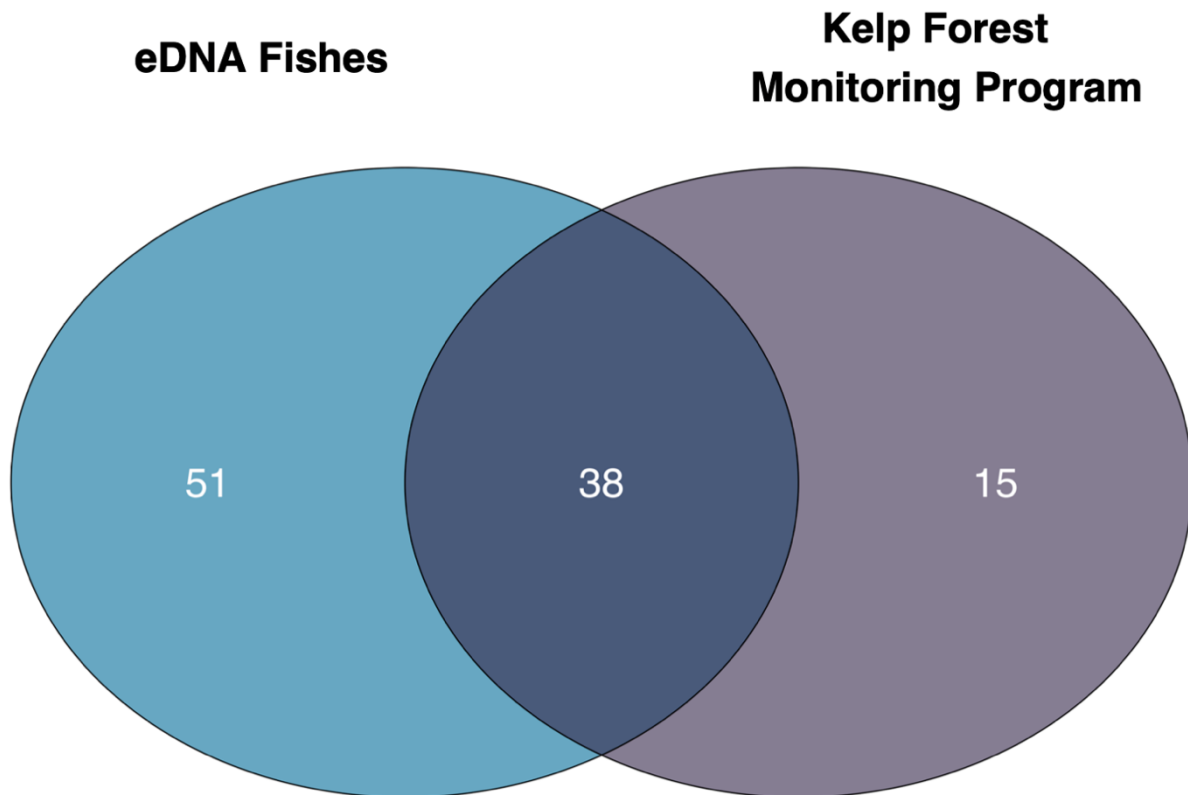
Kelp Forest Monitoring Program surveys recorded 56 fish taxa, representing 53 unique species, 38 genera, 23 families, and 2 classes (Supplemental Table 3-S5) while Reef Check recorded 25 fish taxa (out of 35 monitored total) , representing 23 fish species, 14 genera, 9

families, and 2 classes (Supplemental Table 3-S6). Two of the Reef Check taxa were not observed by the Kelp Forest Monitoring program visual surveys: *Paralabrax nebulifer* and *Sebastes melanops*, while 27 of the species observed by the Kelp Forest Monitoring program are not indicator species monitored by Reef Check.

#### *Comparison of eDNA and Kelp Forest Monitoring Program Surveys*

Across 24 sites, eDNA detected a total of 89 fish species, 37 of which were also visually observed by the Kelp Forest Monitoring Program. The Kelp Forest Monitoring Program also records observations for *Neoclinus* and *Gibbonsia*, but only to the genus level; both were identified by eDNA for a total of 38 shared taxa (Figure 3-10). Kelp Forest Monitoring Program visual surveys observed 15 taxa not detected by eDNA; 12 of these were *Sebastes* (9 species and 3 genus groupings) and the other three were *Lythrypnus dalli*, *Hexagrammos decagrammus*, and *Phanerodon furcatus*. eDNA detected an additional 51 fish species that were not recorded during Kelp Forest Monitoring Program surveys. Of these, 40 have been previously recorded by Kelp Forest Monitoring Program visual surveys.

**Figure 3-10.** Venn Diagram of All Fish Detected by eDNA and Kelp Forest Monitoring Program Visual Dive Surveys Across All Sites



eDNA methods had significantly higher sensitivity than Kelp Forest Monitoring visual fish surveys, roving diver fish count surveys, and fish size frequency surveys [*t*-test ,  $p < 0.0001$ ; eDNA sensitivity: mean 99.5% (98.6-99.7%); Visual fish survey sensitivity: mean 97.2% (93.8-98.7%); Roving diver fish count survey sensitivity: mean 97.6% (91.4-99.1%); Fish size frequency survey sensitivity: mean 95.6% (91.2-96.8%); Table 3-1 ]. In contrast, we found that Kelp Forest Monitoring visual fish surveys, roving diver fish count surveys, and fish size frequency surveys all had significantly higher specificity than eDNA methods [*t*-test,  $p < 0.0001$ ; eDNA specificity: mean 63.5% (60.3-86.2%); Visual fish survey specificity: mean

73.7% (61.6-83.0%); Roving diver fish count survey specificity: mean 73.6% (58.3-96.8%); Fish size frequency survey specificity: mean 85.7% (70.9-94.4%)). We found no significant difference in the sensitivity or specificity of eDNA and 1 m quadrat survey methods, although we note the total sample size of 1 m quadrat fish species observed is only 3 [*t*-test,  $p > 0.1$ ; 1 m quadrat survey sensitivity: mean 97.7% (96.7-99.1%); 1 m quadrat survey specificity: mean 60.6% (56.2-68.4%)].

**Table 3-1.** Average Sensitivity and Specificity for eDNA and Visual Survey Methods.

Maximum and minimum values are reported in parentheses.

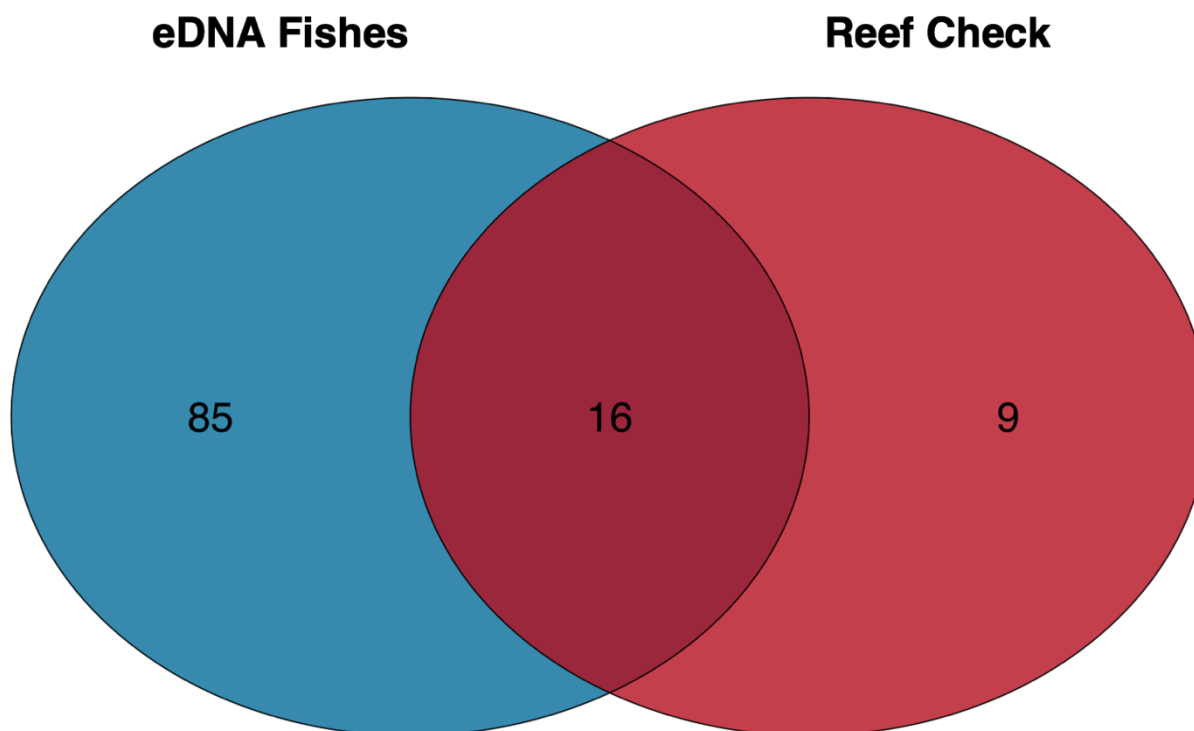
Survey Method	Sensitivity	Specificity
eDNA	99.5% (98.6-99.7%)	63.5% (60.3-86.2%)
Kelp Forest Monitoring Program Visual Fish Transect Surveys	97.2% (93.8-98.7%)	73.7% (61.6-83.0%)
Kelp Forest Monitoring Program Roving Diver Fish Count Surveys	97.6% (91.4-99.1%)	73.6% (58.3-96.8%)
Kelp Forest Monitoring Program Fish Size-Frequency Surveys	95.6 % (91.2-96.8%)	85.7% (70.9-94.4%)
Kelp Forest Monitoring Program 1m Quadrat Surveys	97.7% (96.7-99.1%)	60.6% (56.2-68.4%)
Reef Check	97.2% (89.4-99.6%)	60.8% (51.9-83.0%)

#### *Comparison of eDNA and Reef Check Species Observations*

Across the 28 sites surveyed by both eDNA and Reef Check surveys, eDNA detected a total of 101 fish species of which 16 were recorded by Reef Check surveys (Figure 3-11). Reef Check surveys observed 9 rockfish (*Sebastes* sp.) taxa that eDNA failed to detect, 7 rockfish species and 2 rockfish groups cannot be identified to species level (*S. flavidus*/*S. serranoides* and *Sebastes* young of the year). eDNA detected 85 more species than Reef Check, but only two of

these additional species were indicator species monitored by Reef Check, *Scorpaenichthys marmoratus* and *Sebastes paucispinis*.

**Figure 3-11.** Venn Diagram of All Fish Detected by eDNA and Reef Check Visual Dive Surveys Across All Sites



eDNA methods had significantly higher sensitivity than Reef Check visual survey methods [ $t$ -test,  $p < 0.0001$ ; eDNA sensitivity: mean 99.5% (98.6-99.7%); Reef Check sensitivity 97.2% (89.4-99.6%)]. We found no significant difference in the specificity of eDNA and Reef Check visual survey methods [ $t$ -test,  $p > 0.1$ ; eDNA specificity: mean 63.5% (60.3-86.2%); Reef Check specificity of 60.8% (51.9-83.0%)].

### *eDNA vs. Visual Survey Abundance Estimates*

We found high variability in within-site eDNA index scores as well as within-site biomass and individual count abundance estimates for the majority of species observed. Within-site standard deviations in both eDNA biological and technical replication as well as visual transects spanned over an order of magnitude for many species at any given site. Much of this within-site variation was driven by the lack of detection for a given species between replicate eDNA samples or visual transects within a site.

Correlations between eDNA and visual survey abundance estimates were also highly variable, with R<sup>2</sup> values ranging from 0.00-0.68 (Supplemental Table 3-S7). The highest R<sup>2</sup> values between eDNA and the Kelp Forest Monitoring Program/Reef Check abundance estimates were *Ophiodon elongatus* (R<sup>2</sup> 0.68), *Chromis punctipinnis* (R<sup>2</sup> 0.62), *Oxyjulis californica* (R<sup>2</sup> 0.62), *Embiotoca lateralis* (R<sup>2</sup> 0.53), *Halichoeres semicinctus* (R<sup>2</sup> 0.47), *Heterostichus rostratus* (R<sup>2</sup> 0.52), *Stereolepis gigas* (R<sup>2</sup> 0.41), and *Hypsypops rubicundus* (R<sup>2</sup> 0.41). However, even within these species there was substantial variation between abundance metric, Kelp Forest Monitoring Program survey type, and eDNA vs. Kelp Forest Monitoring Program or vs. Reef Check surveys.

Using biomass instead of numerical abundance did not result in overall improved regressions between eDNA and visual surveys. A few species, such as *Embiotoca lateralis* and *Paralabrax clathratus*, demonstrated improvement in correlation with eDNA index scores when using biomass estimates instead of abundance. Other species, however, such as *Hypsypops rubicundus* and *Chromis punctipinnis*, demonstrated improvement in correlation with eDNA index scores when using counts per transect.

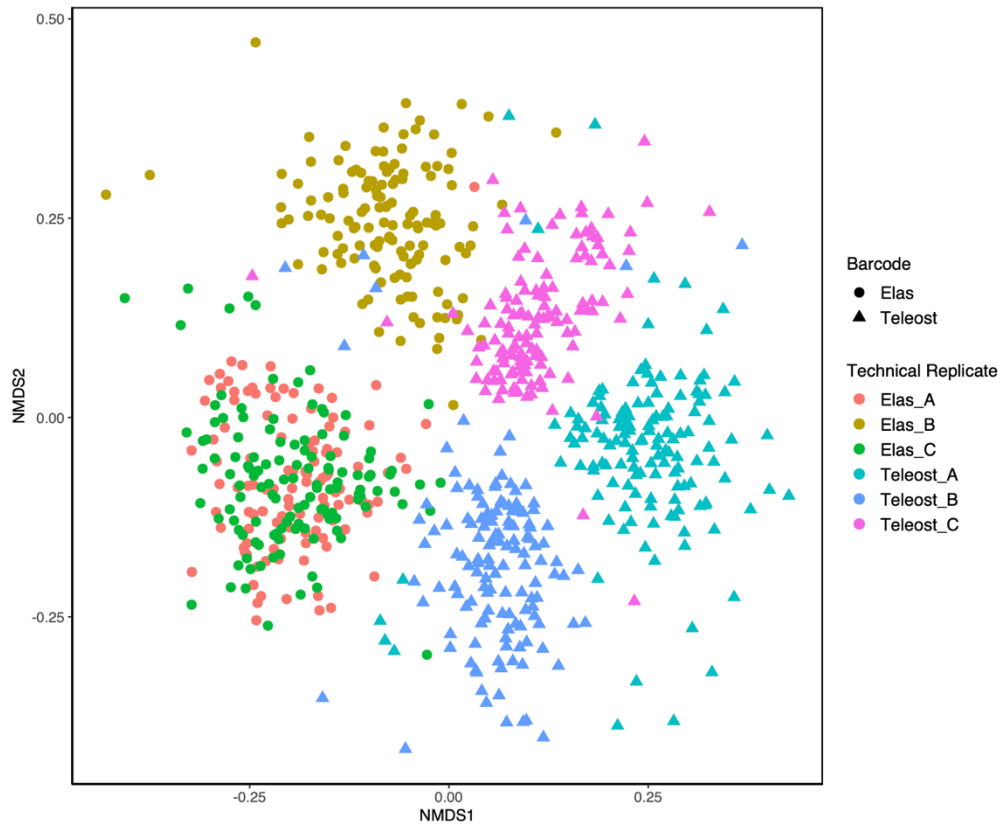


### *Variability Across Levels of Replication*

The nested hierarchical model of eDNA replication explained 95.6% of the observed variance in eDNA samples. The strongest factor was technical replicates (replicate PCR of the same sample), which accounted for 58.6% of the total variation (PERMANOVA  $p < 0.001$ , betadisper  $p = 0.03$ ; Figure 3-12). Teleost vs. elasmobranch specific primer sets only explained 16.2% of the variation (PERMANOVA  $p < 0.001$ , betadisper  $p = 0.001$ ), despite these primers targeting different taxa. We did not find that biological replicates, separate water samples taken at the same site, had a strong effect on structuring of eDNA samples ( $R^2$  9.5%,  $p > 0.5$ ). Site sampled explained 11.3% of the variation observed across eDNA samples (PERMANOVA  $p < 0.001$ , betadisper  $p = 0.027$ ). NMDS ordination reveals strong clustering of technical replicates by sequencing run and barcode, supporting the results of the nested PERMANOVA and homogeneity of dispersion tests (Supplemental Table 3-S8).

**Figure 3-12.** NMDS of Bray-Curtis Dissimilarities between eDNA Technical Replicates.

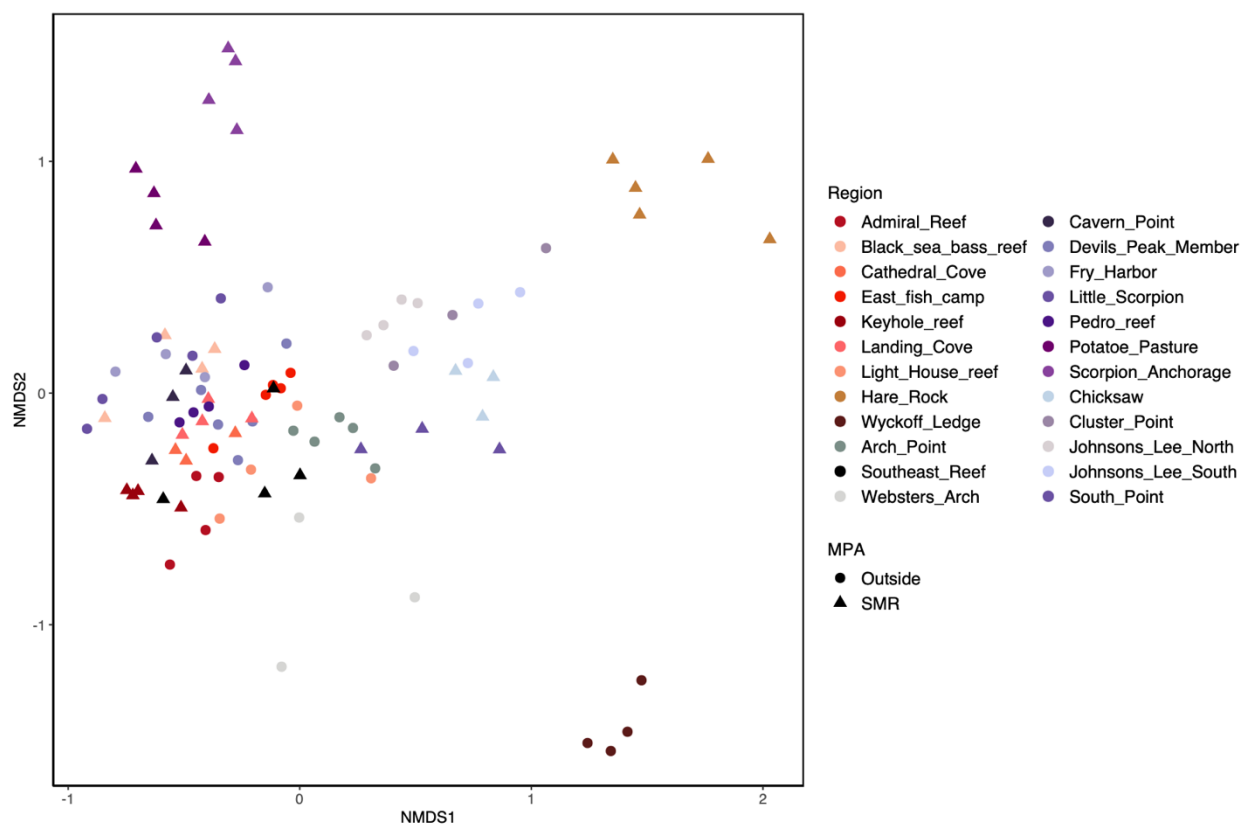
Technical replicates (colors) and barcodes (shapes) are similar to each other (NMDS, Stress = 0.288).



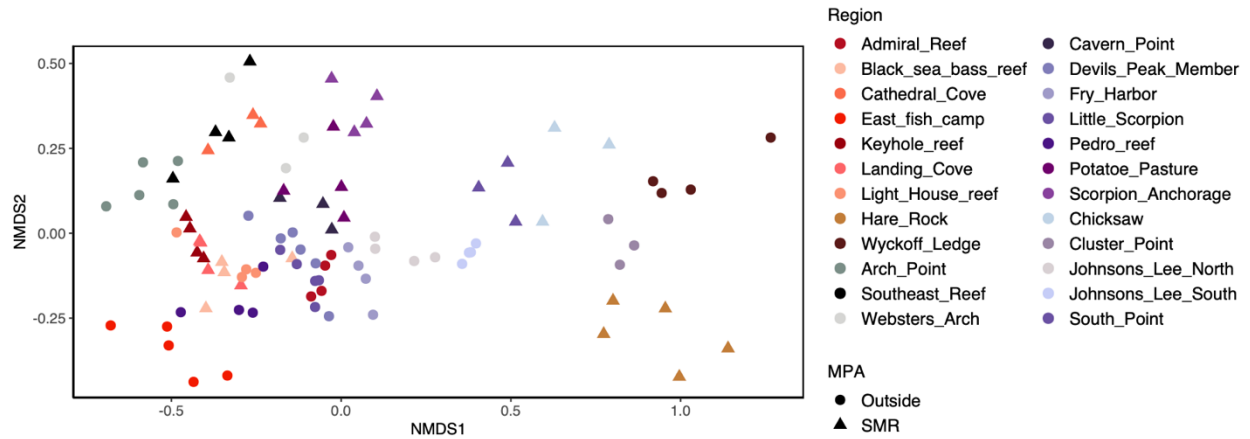
For Kelp Forest Monitoring Program roving diver surveys, the nested hierarchical model of replication explained 87.5% of observed variance. Site was the single strongest factor, explaining 81.1% of the total variation observed (PERMANOVA,  $p < 0.01$ , betadisper  $p < 0.027$ ), followed by transect replicate that accounted for 6.3% of the variation (PERMANOVA  $p = 0.02$ ; Figures 3-13 & 3-14). Neither transect nor site significantly explained the observed variance of visual fish surveys or 1 m quadrat surveys in the nested hierarchical model (PERMANOVA  $p > 0.05$ ; Figures 3-15 & 3-16). However, excluding the nested transects, site alone explained the majority of the variance for both visual fish surveys ( $R^2$ , 60.0%, PERMANOVA,  $p < 0.01$ ,

betadisper  $p = 0.22$ ) and 1m quadrat surveys ( $R^2$  54.5%, PERMANOVA,  $p < 0.01$ , betadisper  $p = .28$ ).

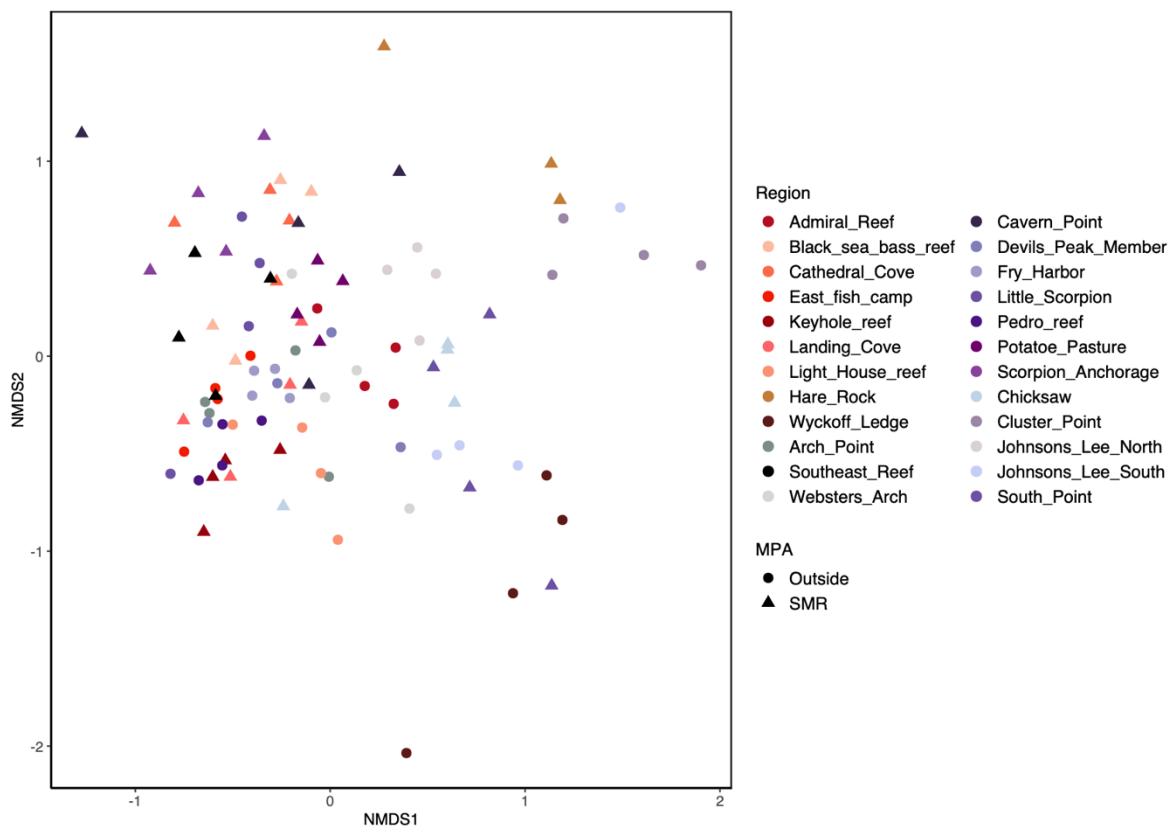
**Figure 3-13.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program Roving Diver Fish Count Survey Transects using Count Data. Region (color) are similar to each other (NMDS, Stress = 0.146). MPA is depicted by shape.



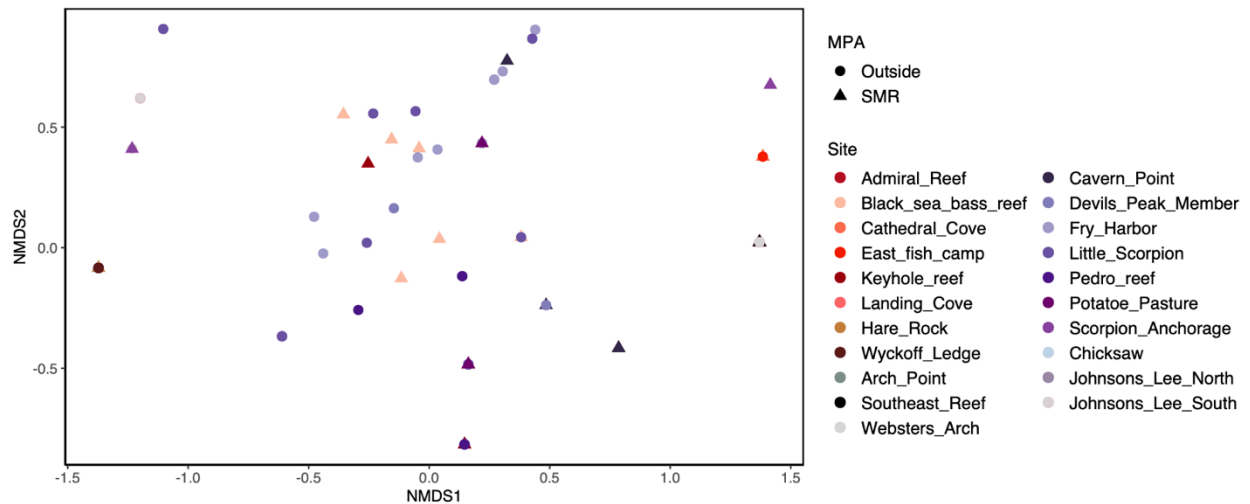
**Figure 3-14.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program Roving Diver Fish Count Survey Transects using Score Data. Region (color) are similar to each other (NMDS, Stress =0.137). MPA is depicted by shape.



**Figure 3-15.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program Visual Fish Survey Transects. Region (color) are similar to each other (NMDS, Stress =0.193). MPA is depicted by shape.

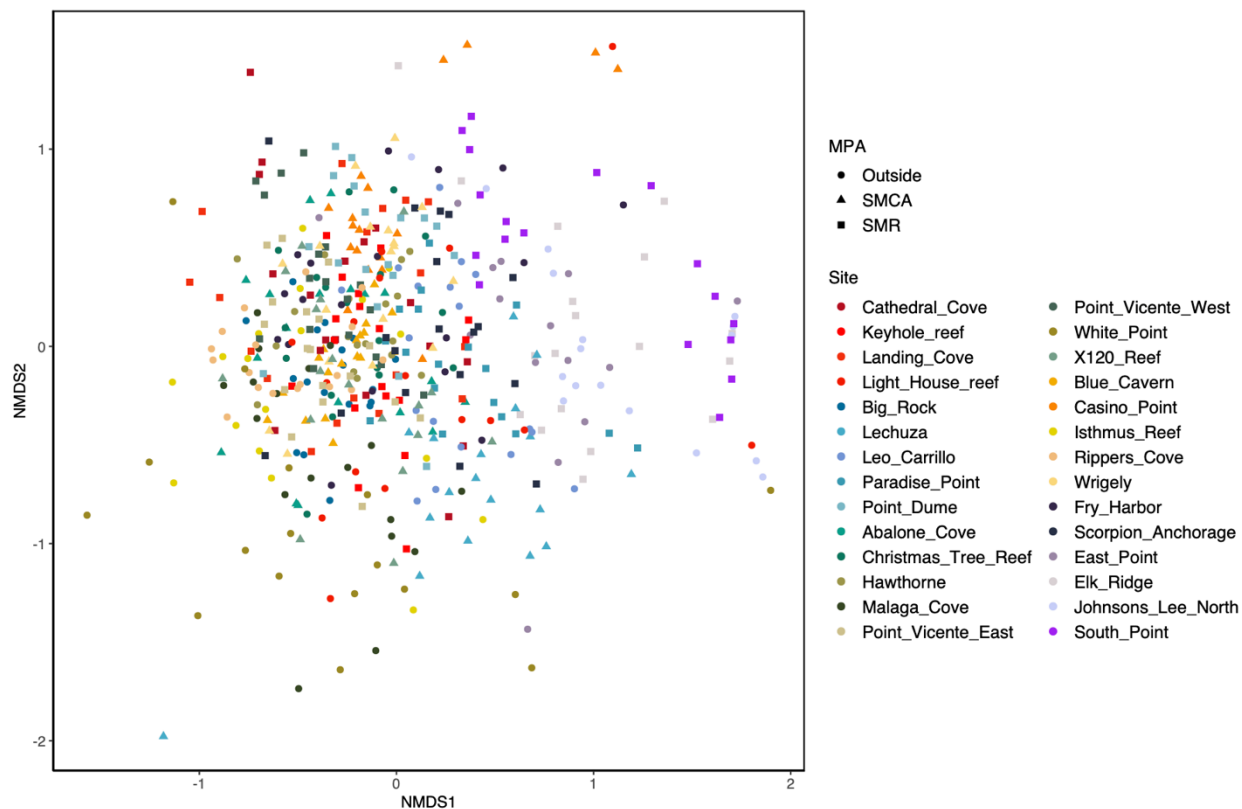


**Figure 3-16.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program 1m Quadrat Survey Transects. Region (color) are similar to each other (NMDS, Stress =0.085). MPA is depicted by shape.

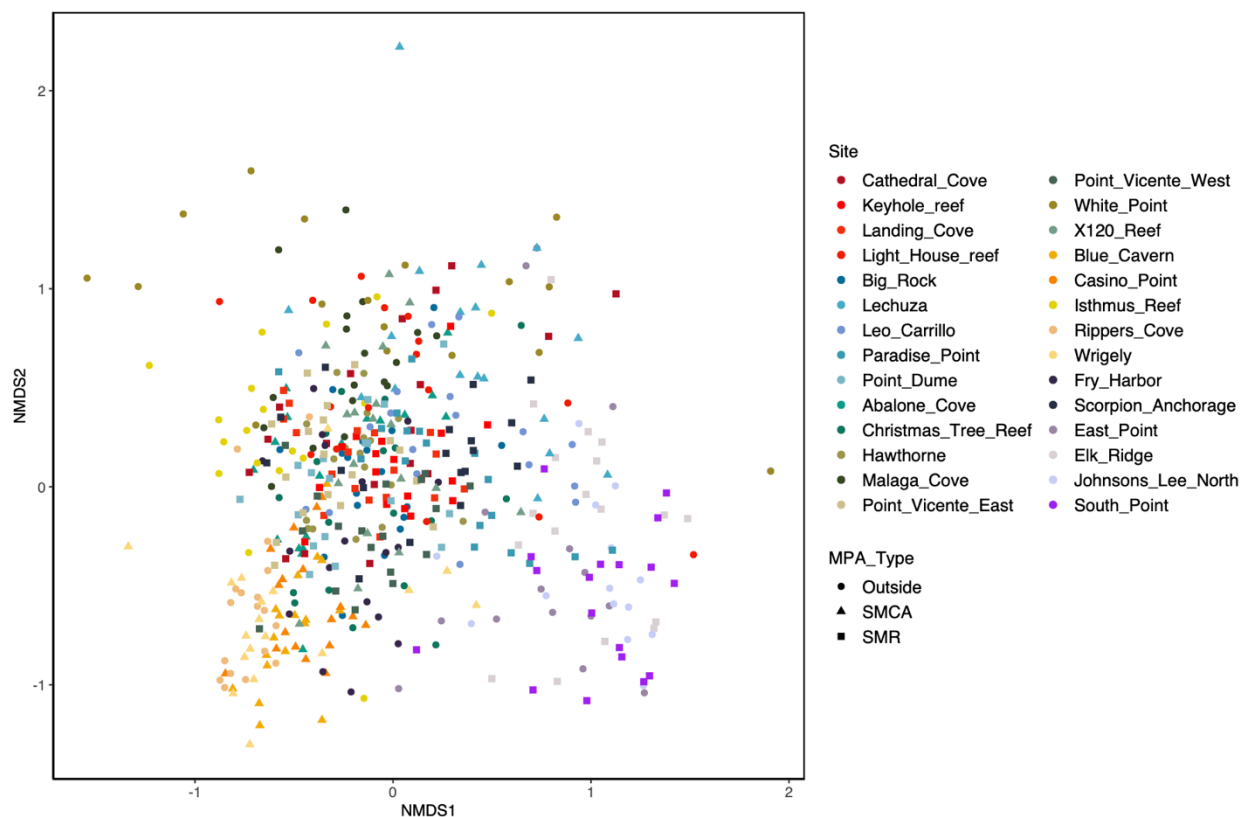


The hierarchical model of replication explained less variation for Reef Check surveys, explaining only 32.7% of the observed variance in biomass. The strongest factor was site, which explained 29.7% of the total variation (PERMANOVA  $p=0.047$ , betadisper  $p << 0.001$ ). Transects did not significantly explain visual survey results ( $R^2$  2.9%,  $p > 0.084$ ; Figures 3-17 & 3-18).

**Figure 3-17.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Survey Transects using Biomass Data. Region (color) are similar to each other (NMDS, Stress =0.239). MPA is depicted by shape.



**Figure 3-18.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Survey Transects using Count Data. Region (color) are similar to each other (NMDS, Stress =0.21). MPA is depicted by shape.



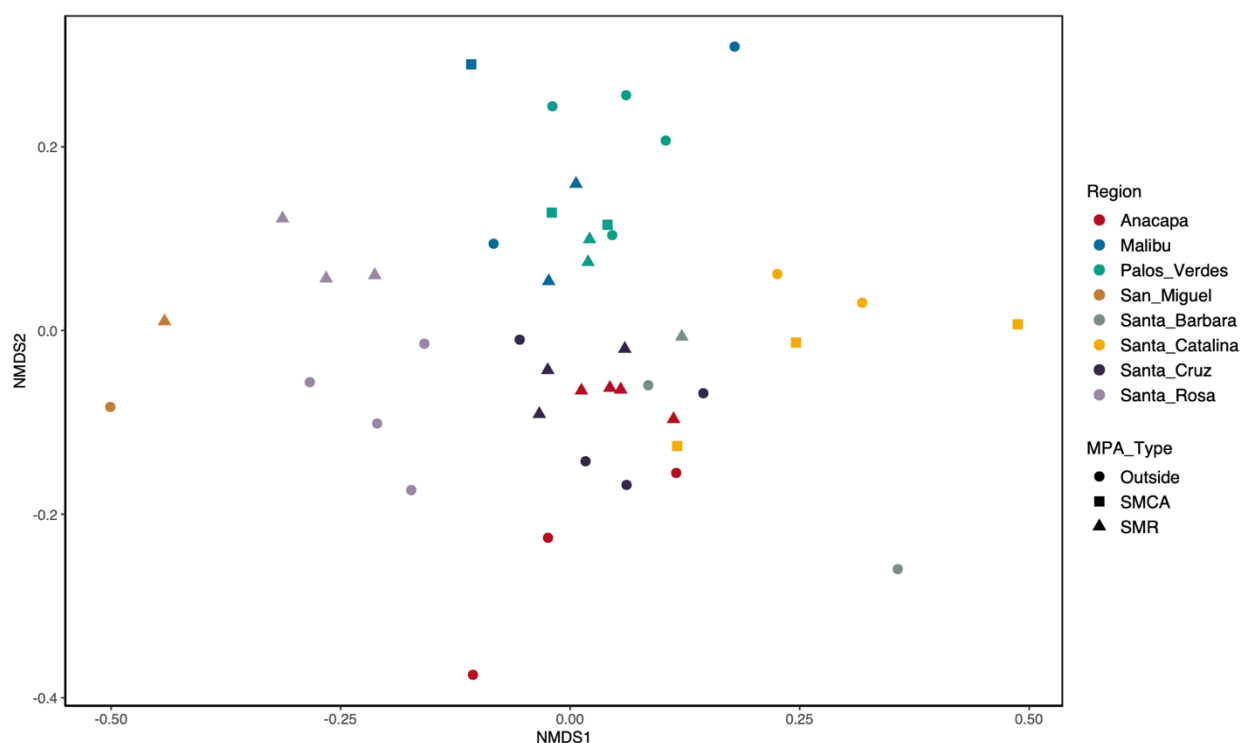
### *Patterns of Community Structure across the MPA Network*

Across all sampled sites and all observed species, eDNA vertebrate community signatures were significantly different across regions ( $R^2$  36.5%, PERMANOVA  $p=0.001$ , betadisper  $p = 0.756$ ), but not inside and outside MPAs ( $R^2$  19.0%, PERMANOVA  $p=0.34$ ; Supplemental Table 3-S9). Comparing only fish detected by eDNA also yielded significant differences among regions ( $R^2$  46.3%, PERMANOVA  $p=0.001$ , betadisper  $p = 0.316$ ) but not inside and outside MPAs ( $R^2$  21.5%, PERMANOVA  $p=0.3$ ). However, when only comparing the 42 taxa indicator taxa observed by both eDNA and visual surveys, we found a significant



difference in fish communities across both regions and inside and outside MPAs (Region - R2 52.9%, PERMANOVA  $p=0.001$ , betadisper  $p = 0.98$ ; MPA - R2 21.2%, PERMANOVA  $p=0.04$ , betadisper  $p = 0.04$ ; Figure 3-19).

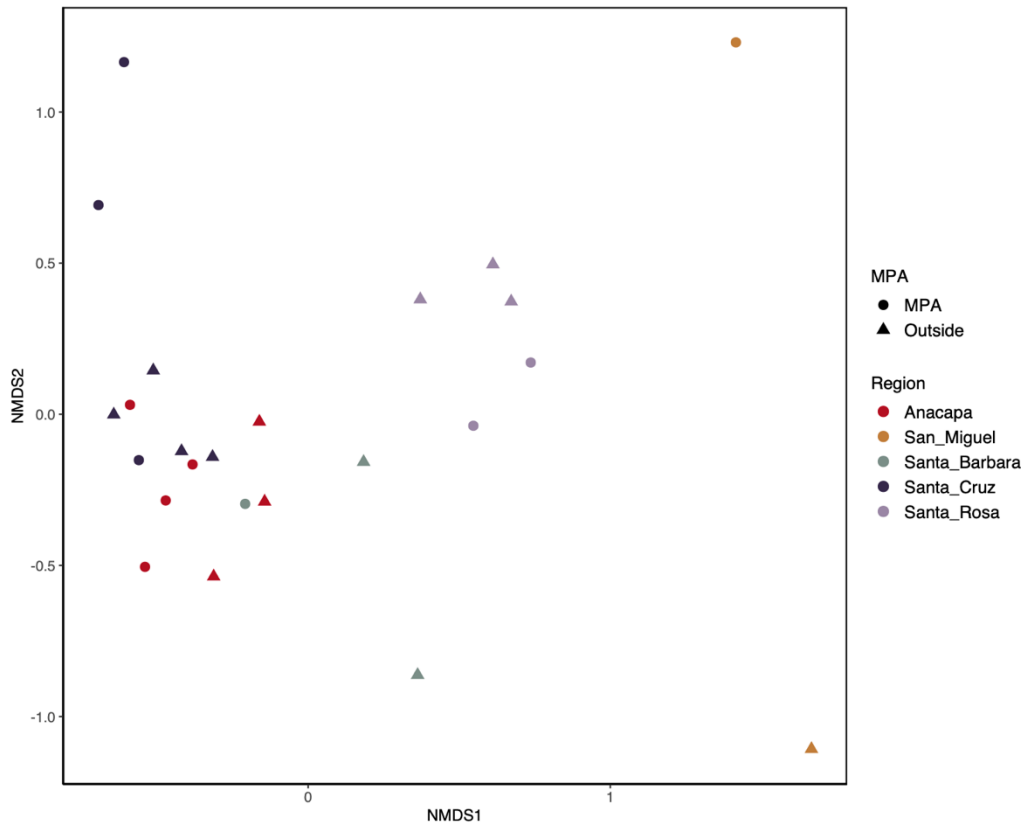
**Figure 3-19.** NMDS of Bray-Curtis Dissimilarities between eDNA Survey Sites using Only Species Observed by Both eDNA and Visual Survey Methods. Region (color) and MPA (shape) are similar to each other (NMDS, Stress =0.198).



Focusing on sites only surveyed by the Kelp Forest Monitoring Program, eDNA found significant differences between regions but not inside and outside MPAs across all species (Region - R2 37.9%; PERMANOVA  $p=0.001$ , betadisper  $p = 0.53$ ; MPA - R2 22.4%, PERMANOVA  $p>0.05$ ). Similar results were obtained when only comparing fishes (Region - R2 37.6%, PERMANOVA  $p=0.001$ , betadisper  $p = 0.29$ ; MPA - R2 22.5%, PERMANOVA

$p > 0.05$ ), but as above, eDNA observed a significant difference in fish species inside and outside MPAs when focused only on species observed by the Kelp Forest Monitoring Program (Region -  $R^2$  47.1%, PERMANOVA  $p = 0.001$ , betadisper  $p = 0.94$ ; MPA-  $R^2$  22.7%, PERMANOVA  $p = 0.04$ , betadisper  $p = 0.16$ ). Kelp Forest Monitoring Program surveys also found significant differences in fish assemblage by region using roving dive survey scores ( $R^2$  69.4%, PERMANOVA  $p = 0.001$ , betadisper  $p = 0.99$ ), roving dive survey counts ( $R^2$  58.5%, PERMANOVA  $p = 0.001$ , betadisper  $p = 0.23$ ), size frequency surveys ( $R^2$  44.7%, PERMANOVA  $p = 0.003$ , betadisper  $p = 0.68$ ), visual fish surveys ( $R^2$  43.7%, PERMANOVA  $p < 0.001$ , betadisper  $p = 0.28$ ), and 1 m quadrat surveys ( $R^2$  58.0%, PERMANOVA  $p < 0.001$ , betadisper  $p = 0.15$ ). The Kelp Forest Monitoring Program did not recover significant differences inside and outside of MPAs except for data from roving diver fish scores inside and outside MPAs (PERMANOVA  $p = 0.045$ , betadisper  $p = 0.93$ ; Figure 3-20).

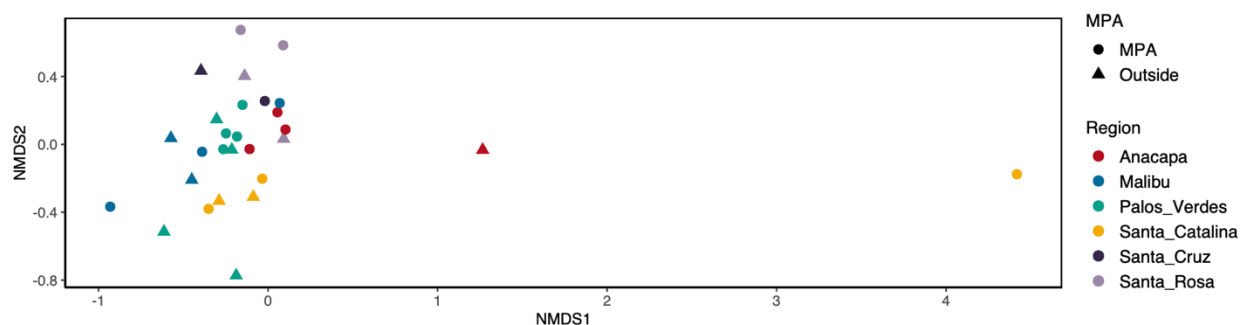
**Figure 3-20.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program Roving Diver Fish Count Survey Sites using Score Data. Region (color) and MPA (shape) are similar to each other (NMDS, Stress =0.091).



At Reef Check sites, eDNA found significant differences across regions for all vertebrate species detected ( $R^2$  55.1%, PERMANOVA  $p = 0.001$ , betadisper  $p=0.38$ ). However, eDNA did not find differences inside and outside MPAs across Reef Check sites (PERMANOVA  $p>0.1$ ). Similar results followed when only comparing all fish species as well as only the 18 taxa observed by Reef visual surveys. These results matched those from Reef Check biomass and count surveys which recorded strong regional differences in fish community structure ( $R^2$  61.3% biomass, PERMANOVA  $p < 0.001$ , betadisper  $p=0.97$ ;  $R^2$  62.0% counts, PERMANOVA  $p <$

0.001, betadisper  $p=0.82$ ) but no differences inside and outside of MPAs (PERMANOVA  $p>0.08$ ; Figure 3-21).

**Figure 3-21.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Survey Sites using Biomass Data. Region (color) and MPA (shape) are similar to each other (NMDS, Stress =0.112).



Given the presence of strong biogeographic patterns across survey sites, we reran comparisons of eDNA and visual monitoring inside and outside MPAs after splitting our sampling sites into 8 subregions. eDNA methods found significant differences in fish communities inside and outside of MPAs in 6 of 8 subregions: Malibu, Palos Verdes, Anacapa Island, Santa Cruz Island, Catalina Island, and Santa Rosa Island, in descending order of MPA effect. These differences were observed across all species, fishes, and only fish observed by both eDNA and visual transect surveys ( $R^2$  6.9-37.4%, PERMANOVA,  $0.001 < p < 0.04$ , betadisper  $0.001 < p < 0.38$ ; Supplemental Table 3-S10; Supplemental Figures S3-1 through S3-7). In contrast, Kelp Forest Monitoring program roving dive count surveys found significant differences in fish communities inside and outside MPAs for 3 of 5 surveyed subregions (Santa Cruz Island, Santa Rosa Island, and Anacapa Island) using both roving fish count methods ( $R^2$  11.7-26.5%, PERMANOVA,  $p = 0.001$ , betadisper  $0.001 < p < 0.976$ ; Supplemental Figures S3-8

through S3-10), but only 2 subregions when using visual fish transects and 1 m quadrat surveys found significant differences in fish communities inside and outside MPAs off Santa Cruz and Anacapa Islands ( $R^2$  5.2-26.0%, PERMANOVA,  $p < 0.02$ , betadisper  $p > 0.38$ ). Reef Check surveys found significant differences in fish communities inside and outside MPAs in 5 of 6 surveyed subregions; Malibu, Catalina Island, Palos Verdes, Santa Rosa Island, and Anacapa Island ( $R^2$  3.7-17.1%, PERMANOVA,  $p < 0.002$ , betadisper  $0.0001 < p < 0.92$ ; Supplemental Figures S3-11 through S3-15).

## **Discussion**

Results highlight the power of eDNA to monitor marine protected areas through the ability to detect significant differences in both fish and vertebrate communities across the Southern California MPA Network. While eDNA and visual survey methods found similar patterns of vertebrate community structure (Hamilton et al. 2010, Zahn et al. 2016), eDNA detected nearly 3 times as many species and detected significant differences in vertebrate communities inside and outside of MPAs network—and did it at a fraction of the cost. Combined, these results contribute to a growing body of evidence of the efficacy of eDNA for monitoring marine ecosystems (Costa et al. 2013, Kelly et al. 2014b, Grorud-Colvert et al. 2014, Caselle et al. 2015).

While eDNA provided a more comprehensive inventory of marine species in Southern California kelp forest habitats and provided a more detailed perspective than underwater visual surveys, it did not always outperform visual surveys. There were key differences between eDNA and visual survey methods in species detected, notably the inability of eDNA to identify rockfish and surfperch to species. In addition, eDNA and visual surveys exhibited differences in relative abundance estimates, variability of replication efforts, and patterns of fish community assemblages across both a biogeographic gradient and inside and outside MPAs (Thomsen et al. 2012, 2016,

Port et al. 2015, Valentini et al. 2016, Ushio et al. 2018a). These results echo previous marine and aquatic eDNA studies that often find substantial differences in observed communities and ecological patterns from eDNA (Kelly et al. 2016, Thomsen et al. 2016).

### *eDNA Detects Broad Array of Marine Vertebrate Diversity*

Previous studies report that eDNA consistently detects more taxa than traditional visual methods (Thomsen et al. 2012, 2016, Kelly et al. 2016, Valentini et al. 2016, Yamamoto et al. 2017, Boussarie et al. 2018, Ushio et al. 2018a). Across all sites, eDNA detected 108 native marine vertebrate taxa, a full 50 more species than observed by Kelp Forest Monitoring program underwater visual surveys which aim to survey all fish species at a given site. We note that comparisons of total observed species between eDNA and Reef Check are unfair given that Reef Check surveys use an intentionally restricted list of key indicator species to allow for increased scope and scale of survey efforts across California (Hodgson et al. 2004). However, the increased breadth of observed vertebrate diversity captured by eDNA compared to Kelp Forest Monitoring program surveys provides the ability to conduct near comprehensive species inventory lists, providing critically important ecological information on a broader subset of nearshore rocky reef communities (Stat et al. 2017, Claisse et al. 2018).

By surveying a larger proportion of marine community, eDNA captures a greater diversity of function groups, trophic guilds, and niches, improving our ability to better understand and describe marine ecosystems and determining the mechanisms and interactions that drive marine community ecology (Steneck et al. 2002, Halpern et al. 2006, Kelly et al. 2014b). Importantly, many of the additional native marine vertebrates detected across the Southern California MPA network are difficult to sample with visual surveys, including highly mobile predators (e.g. Soupfin shark, *Galeorhinus galeus* and Pacific barracuda, *Sphyraena argentea*) as well as species of

important conservation management concern (e.g. Chinook salmon, *Oncorhynchus tshawytscha* and Northern elephant seal, *Mirounga angustirostris*), expanding the scope of MPA monitoring.

In addition to detecting a broader range of species, eDNA had greater sensitivity than both visual survey programs. Notably, eDNA was much more effective in detecting the IUCN red-listed giant black sea bass (*Stereolepis gigas*). Both visual survey programs each recorded *S. gigas* at only one site only—eDNA detected it at 13 sites, including both sites where it was visually observed. The greater ability to detect rare, endangered, or elusive species, is a major advantage of eDNA as it can provide a more sensitive monitoring tool for estimating population size, even when just assuming presence-absence detections (Chabot et al. 2015, House et al. 2016, Benseman et al. 2019).

Despite detecting a much wider breadth of marine vertebrate taxa, eDNA failed to identify key indicator species, notably *Sebastes*. This result, however, is not an inherent shortcoming of eDNA. Instead, it stems from the relatively slow rate of evolution in the *12S* MiFish barcode region, resulting in insufficient genetic diversity (Miya et al. 2015, Yamamoto et al. 2016) to differentiate among the 67 species within this recent radiation that inhabit the Pacific Coast of North America (Ingram and Kai 2014a). Given the ecological (Lea et al. 1999) and economic (Williams et al. 2010) importance of *Sebastes* and the fact that rockfishes accounted for 23-56% of indicator species recorded by the two visual survey programs in this study, the inability to resolve *Sebastes* species is a major shortcoming.

Like rockfish, surfperches are also a recent adaptive radiation (Hyde and Vetter 2007, Longo 2016) that exhibits limited genetic diversity in the *12S* locus (Longo and Bernardi 2015). Taxonomic assignment is further hindered by the high number of different genera assigned to these closely related fishes (Longo et al. 2018). Many bioinformatic pipelines use “lowest

common ancestor” classification schemes to assign taxonomy (Gao et al. 2017). The surfperches *Phanerodon furcatus* and *Zalembeius rosaceus* have identical MiFish 12S barcodes, so the lowest common ancestor methods default to family level assignments. Across California surfperches, there are 7 distinct clades of which two have matching barcodes. Moreover, in addition to the above surfperches, *Amphistichus argenteus*, *Amphistichus koelzi*, *Amphistichus rhodoterus*, *Hyperprosopon anale*, *Hyperprosopon argenteum*, and *Hyperprosopon ellipticum* also have identical barcodes.

This inability of eDNA to distinguish among surfperches (Embiotocidae) and rockfishes (*Sebastes* sp.) should not, however, be viewed as an indictment of the utility of eDNA. Rather, it highlights important taxa specific limitations of the MiFish 12S primers set (Miya et al. 2015), an issue that can likely be overcome by using novel, complimentary barcoding primers (Thompson et al. 2017, Duke and Burton 2020).

#### *High Variability Across eDNA and Visual Transect Surveys*

Both eDNA and visual surveys exhibited both high between-method and within-site variability with respect to species detections and abundance estimates. Comparing species detections across sites showed that eDNA and visual methods failed to capture species observed by the other method, suggesting substantial mismatch between these methods (Kelly et al. 2017). However, this discordance was often the result of the inability of MiFish primers to accurately distinguish among *Sebastes* and surfperches (above).

Interestingly, eDNA failed to detect some commonly observed species that are easily classified using eDNA metabarcoding (Pinfield et al. 2019). For example, in 4 cases, eDNA didn’t detect Garibaldi (*Hypsypops rubicundus*), even though it was observed in Kelp Forest Monitoring Program visual surveys. Given the high site fidelity of this species we would expect



the temporal difference in sampling to have little effect on eDNA detections (Caron and Rainboth 1992, Knapp et al. 1995). This result is reflected in the higher estimated true positive rate of Kelp Forest Monitoring program visual surveys (92.9%) compared to eDNA methods (45.5%) for this species. High true positive rates for visual surveys are unsurprising given the fluorescent bright orange coloration of the species, but low true positive rates for eDNA are concerning given how common the species is.

However, such false negatives are not unique to eDNA methods—visual surveys frequently fail to detect common taxa as well (Hodgson et al. 2004). For both methods, these false negatives can be attributed to the stochastic nature of fish behavior and limited survey time can lead to chance detection failures (Bernard et al. 2013).

To reduce false negatives, the Kelp Forest Monitoring Program has shifted to longer roving diver surveys while Reef Check has added high spatial replication of fish transect surveys. Likewise, eDNA sampling strategies could maximize species detections by pooling multiple water samples taken at slightly different locations or times. The ease of sample collection, potential for automation, and low cost of eDNA methods should allow eDNA methods to be easily scaled up, the resulting increase in sample size reducing variability in site-species detections (Good et al. 2018, Yamahara et al. 2019).

Relative abundance estimates of both eDNA and visual transect surveys also exhibited high between-method and within-site variation. Previous studies report a modest relationships between abundance estimates from eDNA and traditional survey methods (Thomsen et al. 2016, Ushio et al. 2017, Shelton et al. 2019). Likewise, we found modest abundance relationships for a few taxa, but these relationships were inconsistent. In some cases, species with high eDNA index scores were

not observed at all in visual surveys and vice-versa as previously reported in comparisons of eDNA and visual survey methods (Port et al. 2015, Thomsen et al. 2016, Kelly et al. 2017).

Furthermore, eDNA and visual surveys had high variability between replicate transects/samples often spanning an order of magnitude (Bernard et al. 2013, Fonseca 2018). As discussed above, frequently only one or two replicate measures would successfully detect a common species at site for both eDNA and visual survey methods. For eDNA sampling, the variability in abundance estimates and ability to detect a species has been shown to be predominantly driven by under sampling of both water samples as well as technical replicates (Chambert et al. 2018, Doi et al. 2019). In contrast, high within-site variability in visual surveys is due to both fish association with specific locations on a reef as well as under sampling of reef habitats (Edgar et al. 2004, Bernard et al. 2013, Mireles et al. 2019). Our current study cannot disentangle the relative importance of within-site fish association and under sampling of visual surveys. However, based on previous comparisons of visual fish surveys our results suggest that visual surveys are also under sampling marine environments to some degree (Edgar et al. 2004, Bernard et al. 2013).

The high within-site variability in both eDNA and visual survey abundance methods are concerning as abundance metrics are critical to informing the success of the Southern California MPA network, and for providing mechanistic understandings of ecosystem functioning during global change (Hamilton et al. 2010, Pondella II et al. 2015, 2019, Claisse et al. 2018). Our findings suggest that accurately capturing abundance estimates within a kelp forest ecosystem requires a substantial increase in what is already a tremendous effort and use of resources for visual surveys, especially to measure rare and endangered species that are infrequently observed and monitored (Chao et al. 2014). Given the cost effectiveness and ease of sample collection of sea

water samples (Gold *et al.* in prep), eDNA methods can provide an added-benefit to increase sampling effort of these marine communities at lower cost than expanding visual monitoring survey programs (Kelly *et al.* 2014b). Furthermore, advancements in eDNA methods including internal-standards have been found to substantially improve abundance estimates (Ushio *et al.* 2018a, 2018b). Improvements in eDNA methods and the ability to relatively easily increase eDNA sampling efforts suggests it can be an effective method to quantify fish abundances, even if only used in a presence/absence site occupancy modeling approach (Lacoursière-Roussel *et al.* 2016, Chambert *et al.* 2018).

### *Community Structure across the MPA Network*

Previous studies of the Southern California MPA Network indicate fish communities are shaped more strongly by geography than by being in MPAs (Hamilton *et al.* 2010, Claisse *et al.* 2018), patterns recovered in this study by both eDNA and visual survey protocols. The ability of eDNA to capture turnover in fish communities across biogeographic borders indicates that eDNA is scalable, allowing researchers to survey more marine ecosystems, more frequently and gain better insights into community assembly patterns over broader spatial scales.

Importantly, eDNA also detected significant differences in fish communities inside and outside of individual MPAs in the Southern California MPA Network. For example, eDNA Index scores indicated higher abundances of *Semicossyphus pulcher*, *Caulolatilus princeps*, and *Embiotoca jacksoni* inside Santa Cruz and Anacapa MPAs compared to outside. Similarly, eDNA Index scores were higher for *Ophiodon elongatus*, *Embiotoca jacksoni*, and *Scorpaenichthys marmoratus* in South Point State Marine Reserve off Santa Rosa Island compared to outside the reserve. In addition, the two MPAs in Malibu also had higher eDNA index scores of *Semicossyphus pulcher*, *Girella nigricans*, and *Sphyrna argentea*. Importantly, all of the above

taxa are recreational fisheries targets that are known to increase in abundance after the establishment of MPAs (Pondella II et al. 2015, Caselle et al. 2015, Starr et al. 2015).

An interesting result of this study was that eDNA found stronger differences inside and outside MPAs among fish species observed by visual survey programs than for entire fish communities. Given that the indicator species were selected because they play important ecological roles in kelp forest ecosystems and are assumed to represent kelp forest ecosystem health, it is perhaps unsurprising these species indicate shifts in fish community structure across MPAs. Importantly, eDNA could discriminate among MPA and non-MPA sites despite the inability of eDNA to differentiate among the 14 rockfish species observed in visual surveys. This result suggests that even when missing data on a third of indicator species targeted by visual surveys, eDNA is still capable of providing important information on fish communities inside and outside of MPAs.

#### *eDNA Metabarcoding is A Very Different Kind of Survey*

Studies, like the present, focused on understanding the utility of eDNA approaches use visual observations as the “gold standard”. However, such comparisons are fraught with challenges (Goldberg et al. 2016, Deiner et al. 2017) as both approaches have unique biases. Visual methods are biased by observer limitations and tend to act in consistent and relatively well understood manner (Bernard et al. 2013, Lindfield et al. 2014). In contrast, eDNA biases are often driven by PCR biases which act in stochastic and compounding ways that are much less well characterized (Goldberg et al. 2016, Gibbons et al. 2018, Kelly et al. 2019, Mata et al. 2019). Many of eDNA biases derive from what makes eDNA such a promising tool—it’s tremendous sensitivity as demonstrated here. This sensitivity allows eDNA to detect species occurrences far beyond what is possible in visual surveys, but also increases the potential for false-positives from processes

happening both in the field and lab (Pedersen et al. 2015, Chambert et al. 2015, Goldberg et al. 2016, Deiner et al. 2017, McKnight et al. 2019). Furthermore, eDNA methods are strongly influenced by the generation, degradation, and transport of eDNA, leading to differences in integrated time and space captured by eDNA methods that may bias eDNA methods in unique ways compared to visual surveys (Hodgson et al. 2004, Sprague et al. 2013, Port et al. 2015, Yamamoto et al. 2016, Sassoubre et al. 2016, Barnes and Turner 2016, Andruszkiewicz et al. 2017a, 2019, Kelly et al. 2018, Murakami et al. 2019, Shelton et al. 2019).

That eDNA has any biases does not mean it is inferior to visually based survey protocols. The difference is that visual approaches have been used for decades, allowing time for these biases to become well understood (Edgar et al. 2004, Bernard et al. 2013), for the development strategies to mitigate those biases (Lindfield et al. 2014), and for the scientific community to accept these methods despite known biases (Lessios 1996, Edgar et al. 2004, Bernard et al. 2013). Similarly, broadscale adoption of eDNA methods hinges on understanding and controlling biases, and using decades of analytical and statistical frameworks to develop approaches to control biases and the variation they introduce (Schmidt et al. 2013, Chambert et al. 2018, Doi et al. 2019). However, all methods have biases—as such, the success of eDNA methods should not be judged solely on the comparison to visual sampling efforts. Instead, it's important to understand the advantages and disadvantages of different approaches so that studies are designed to draw on the strength of a methodology, while accounting for their weaknesses. In some cases, eDNA and visual surveys may be used complementarily, much like visual surveys and bait remote underwater video surveys (BRUVs) (Langlois et al. 2010).

## **Conclusion**

The results of this study highlight the power of eDNA to provide important data on kelp forest fish communities, data that is essential for monitoring the health of the Southern California MPA network (Pondella II et al. 2015) and the effectiveness of current management practices. Importantly, eDNA provides marine resource managers and researchers the opportunity to expand the taxonomic scope and geographic scale of their surveys, allowing them to address important fundamental ecological questions on community assembly in dynamic marine ecosystems, especially in response to global change (Bohmann et al. 2014).

While eDNA is opening up new windows into understanding marine ecosystems, particularly MPAs, there will always be value in having divers in the water observing the ecosystem. However, because eDNA is easy, rapid and economical, large monitoring programs like the Kelp Forest Monitoring Program and Reef Check could easily include eDNA in their monitoring activities to provide the strongest data possible to effectively monitor and manage the Southern California MPA Network, ensuring the long term sustainability of these critically important coastal ecosystems.

## CONCLUSION

Marine ecosystems worldwide are increasingly under threat from local and global anthropogenic stressors (Tegner et al. 1995, Jackson et al. 2001, Fabry et al. 2008, Diaz and Rosenberg 2008, Doney et al. 2009, 2012, Hoegh-Guldberg and Bruno 2010b, Bolster, Jeff et al. 2012), thus effective and efficient monitoring of marine communities is of critical importance to inform dynamic ocean management (Maxwell et al. 2015). Combined, the results of my dissertation highlight the power of eDNA to provide important monitoring information on California Current fish communities both providing a broad range of accurate species detections as well as fish community responses to biogeographic patterns and MPAs. These results demonstrate that eDNA metabarcoding can provide essential information for the continued monitoring the health of the Southern California MPA network (Pondella II et al. 2015). This characterization of marine fish communities across the Southern California MPA network through eDNA metabarcoding methods is timely as the California State Ocean Protection Council is in the process of evaluating the effectiveness of this MPA network (Saarman et al. 2013). Specifically our eDNA results align well with visual surveys and previous studies demonstrating that many of the Southern California MPAs are working, having significantly different fish communities inside MPAs including higher abundance of some target marine fish species (Pondella II et al. 2015, Hamilton and Caselle 2015, Zellmer et al. 2018). Furthermore, these eDNA data provide results on a much broader taxonomic scope of marine fishes across the Southern California MPA network demonstrating that changes in fish communities are not just limited to the limited species observed in visual surveys.

eDNA metabarcoding approaches will continue to improve as our understanding of the ecology eDNA becomes more refined and our analytical approaches to process and generate eDNA become refined. In particular, efforts to adapt eDNA metabarcoding approaches to population genetics has the potential to dramatically expand our ability to characterize marine connectivity, population dynamics, and behavioral ecology (Barnes and Turner 2016, Sigsgaard et al. 2017, Stat et al. 2017, Parsons et al. 2018). In addition, future research into the fate and transport of eDNA in marine systems will improve our interpretation and application of eDNA methods for biomonitoring.

Given the ease of sample collection, the potential for rapid and automated data generation, and cost effectiveness of eDNA methods, large scale marine ecosystem monitoring programs should consider including eDNA metabarcoding to provide low cost, value added marine ecosystem assessment capabilities, dramatically expanding marine ecosystem monitoring across space, time and depth. Importantly, the ease of eDNA sample collection facilitates the use of community scientist volunteers and can allow for dramatic increases in spatial and temporal coverage of biomonitoring surveys for a fraction of the cost of employing professional divers (Gillett et al. 2012, Biggs et al. 2014, Spear et al. 2017, Deiner et al. 2017, Kandlikar et al. 2018, Freiwald et al. 2018, Meyer et al. 2019). For example, before the corona virus pandemic, we worked with Heal the Bay's MPA Watch community science program to conduct monthly eDNA monitoring of 4 coastal rocky reefs off Malibu, CA, detecting over a hundred of species of marine vertebrates each month at a cost of \$10,000 (data not shown).

Ignoring the significant economies of scale of employing eDNA methods, a rough estimate to conduct monthly surveys inside and outside all California MPAs would be under \$1.3 million or 0.5% of annual ex-vessel commercial fisheries values (Miller et al. 2017). These monthly surveys



of nearshore marine ecosystems would dramatically expand the taxonomic scope, geographic scale, and temporal resolution of marine ecosystems surveys. This increased resolution of marine ecosystem assessments could provide marine resource managers and researchers the critical information needed to adequately manage our marine ecosystems in response to global climate change and help resolve key ecological questions on the assembly and dynamics of marine communities (Bohmann et al. 2014).

## SUPPLEMENTAL CHAPTER 1

### *Anacapa Toolkit:*

#### **an environmental DNA toolkit for processing multilocus metabarcode datasets**

#### **Abstract**

1. Environmental DNA (eDNA) metabarcoding is a promising method to monitor species and community diversity that is rapid, affordable, and non-invasive. Longstanding needs of the eDNA community are modular informatics tools, comprehensive and customizable reference databases, flexibility across high-throughput sequencing platforms, fast multilocus metabarcode processing, and accurate taxonomic assignment. Improvements in bioinformatics tools makes addressing each of these demands within a single toolkit a reality.
2. The new modular metabarcode sequence toolkit *Anacapa* (<https://github.com/limey-bean/Anacapa/>) addresses the above needs, allowing users to build comprehensive reference databases and assign taxonomy to raw multilocus metabarcode sequence data. A novel aspect of *Anacapa* is its database building module, “Creating Reference libraries Using eXisting tools” (*CRUX*), which generates comprehensive reference databases for specific user-defined metabarcoding loci. The *Quality Control and ASV Parsing* module sorts and processes multiple metabarcoding loci and processes merged, unmerged and unpaired reads maximizing recovered diversity. *DADA2* then detects amplicon sequence variants (ASVs) and the *Anacapa Classifier* module aligns these

ASVs to *CRUX*-generated reference databases using *Bowtie2*. Lastly, taxonomy is assigned to ASVs with confidence scores using a Bayesian Lowest Common Ancestor (*BLCA*) method. The *Anacapa Toolkit* also includes an R package, *ranacapa*, for automated results exploration through standard biodiversity statistical analysis.

3. Benchmarking tests verify that the *Anacapa Toolkit* effectively and efficiently generates comprehensive reference databases that capture taxonomic diversity and can assign taxonomy to both MiSeq and HiSeq-length sequence data. We demonstrate the value of the *Anacapa Toolkit* in assigning taxonomy to seawater eDNA samples collected in southern California.
4. The *Anacapa Toolkit* improves the functionality of eDNA and streamlines biodiversity assessment and management by generating metabarcode specific databases, processing multilocus data, retaining a larger proportion of sequencing reads, and expanding non-traditional eDNA targets. All the components of the *Anacapa Toolkit* are open and available in a virtual container to ease installation.

## 1 | Introduction

Rapid and inexpensive biodiversity monitoring tools are critical for maintaining healthy ecosystems and for effective species conservation (Deiner et al. 2017). Environmental DNA (eDNA) is a promising non-invasive approach for biodiversity monitoring that is increasingly used in ecology and conservation research. Although eDNA metabarcoding is a powerful, rapid and cost-effective approach to survey taxa in terrestrial and aquatic ecosystems (Taberlet et al. 2012b, Kelly et al. 2014a, Bohmann et al. 2014, Deiner et al. 2017), three key bioinformatics challenges in sequence processing and taxonomic assignment limit the accuracy and reliability of eDNA approaches.

First, to capture a broad representation of taxonomic diversity, many eDNA studies simultaneously sequence multiple loci per sample (e.g. (Stat et al. 2017)). However, few metabarcode pipelines are explicitly designed to process multilocus high-throughput sequencing data (but see (Arulandhu et al. 2017)). As such, researchers must sort and process multiple eDNA metabarcodes independently, substantially increasing computation time with each additional metabarcode.

A second challenge for eDNA metabarcode processing is the lack of robust, locus-specific reference databases (Deiner et al. 2017). Curated databases for select metabarcoding loci offer validated solutions for certain commonly-used universal metabarcodes (e.g. UNITE for Fungal *ITS* sequences, (Kõljalg et al. 2013)), but such curated databases are unlikely to exist for all loci used in metabarcoding studies, especially as the number of target metabarcodes grows. Custom, user-generated databases are a promising solution, but current approaches can be problematic. For example, generating reference databases through *in silico* PCR will miss reference sequences that do not contain primer recognition sites, a feature of many Sanger-based sequences (Ficetola et al. 2010, Boyer et al. 2016). Furthermore, methods that rely on keyword searches to generate reference databases are sensitive to inaccurate metadata (Machida et al. 2017) and are susceptible to retrieving sequences that lack the target metabarcode locus. Together, these issues highlight a need for a more comprehensive reference databases to enhance eDNA metabarcoding taxonomic assignment.

A third challenge of existing metabarcode pipelines is that they frequently discard large portions of sequence data, including reads that can be valuable for assigning taxonomy. For example, some pipelines discard unmerged sequences entirely, or only use partial sequence data where full-length alignment with reference metabarcodes is not possible (Port et al. 2015)

potentially causing selection bias against certain taxa (Deagle et al. 2014). To attempt to solve this issue, some pipelines employ nested least common ancestor assignments to non-contiguous sequences (See (Huson and Weber 2013)), but the lack of joint assignment limits the achievable taxonomic resolution. Few pipelines are specifically designed to handle unmerged paired data (e.g. (Bengtsson-Palme et al. 2015)), relying heavily on BLAST to assign taxonomy. However, both these approaches usually limit the number of BLAST hits returned, which presents an additional problem because BLAST will prioritize the sequence order within the reference database over the best alignment for taxonomic assignment (Shah et al. 2019). Furthermore, improved handling of unmerged paired sequences would enable researchers to more readily leverage new high-throughput sequencing platforms (e.g. Illumina NovaSeq and 10X) and barcoding loci of longer length (Deiner et al. 2017).

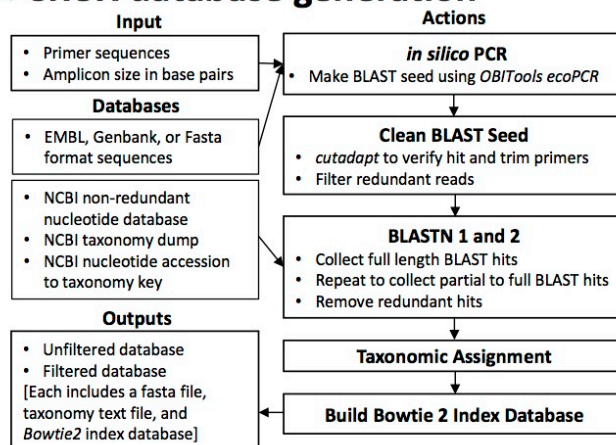
To help resolve these challenges, we developed the *Anacapa Toolkit*, a bioinformatic pipeline with modules for: 1) creating custom reference databases; 2) executing quality control and multilocus read parsing; 3) generating taxonomic assignments for all quality reads produced by HiSeq and MiSeq Illumina platforms; and 4) interactively visualizing taxonomy tables from the *Anacapa Toolkit* using the R package *ranacapa* as described in (Kandlikar et al. 2018).

## **2 | *The Anacapa Toolkit***

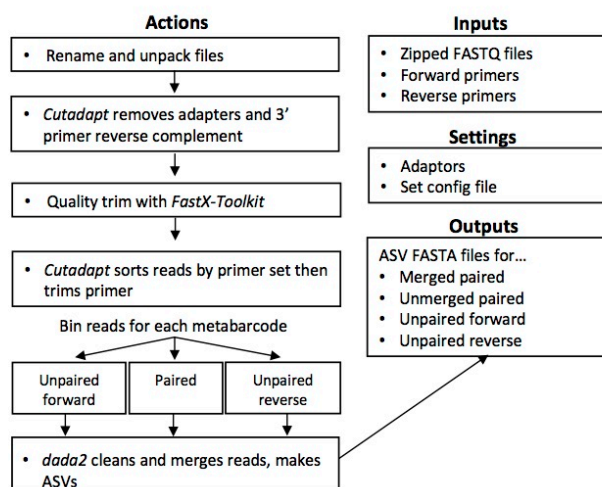
The *Anacapa Toolkit* combines components of leading bioinformatics software with custom methods (Figure S1-1). The first module, Creating Reference libraries Using eXisting tools (*CRUX*), generates custom reference databases. The second module performs raw sequence quality control and employs *DADA2* (Callahan et al. 2016) to infer Amplicon Sequence Variants (ASVs). The third module assigns taxonomy using *Bowtie2* and the Bayesian Lowest Common Ancestor algorithm (*BLCA*; (Gao et al. 2017)).

Figure S1-1. Flowchart of the *Anacapa Toolkit*

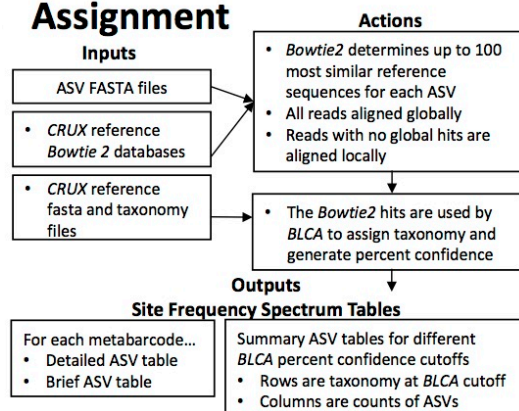
## A. CRUX database generation



## B. Sequence QC and ASV Parsing



## C. Assignment



## D. Exploration with *ranacapa*

All components of the *Anacapa Toolkit* are openly available (<https://github.com/limey-bean/Anacapa>) and archived in DRYAD (<https://doi.org/10.5061/dryad.mf0126f>). Appendices 1-6 referred to in this Supplemental Chapter are available online at <https://doi.org/10.1111/2041-210X.13214>. The *Anacapa Toolkit* and several *CRUX*-generated reference databases are available in virtual containers developed with Code for Science and Society (Ogden 2018). A detailed list of all parameters and their functions is presented in Appendix 1.

## **2.1 | *CRUX*: Creating Reference libraries Using eXisting tools**

The *Anacapa Toolkit*'s first module, *CRUX* (Figure S1-1A; Appendix 1.1), constructs custom reference databases for user-defined primers by querying public databases. *CRUX* first generates metabarcode-specific seed databases by running *in silico* PCR (Ficetola et al. 2010) on the EMBL standard nucleotide database (Stoesser et al. 2002). To increase the breadth of reference sequences and capture sequences without barcode primers, *CRUX* then uses *blastn* (Camacho et al. 2009) to query the seed databases against the NCBI non-redundant nucleotide database (Pruitt et al. 2005). *CRUX* de-replicates the *blastn* hits by retaining only the longest version of each sequence and retrieves taxonomy using *Entrez-giime* (Baker 2016). For each primer set, *CRUX* generates an “unfiltered” database that contains all accessions and taxonomic paths, a paired “filtered” database that excludes accessions with ambiguities in the taxonomic paths, and a *Bowtie2*-formatted index library (Langmead and Salzberg 2012).

## **2.2 | Sequence Quality Control and ASV Parsing**

The *Anacapa Toolkit*'s *Quality Control and ASV Parsing* module (Figure S1-1B; Appendix 1.2) conducts standard DNA sequence quality control and generates ASVs. It uses *cutadapt* (Martin 2011) and *FastX-toolkit* (Gordon and Hannon 2010) to trim user-defined

primers and adapters and low-quality bases from raw FASTQ files from Illumina sequencing platforms. Next, this module uses *cutadapt* to separate reads from multiple loci within each sample. A custom Python script sorts locus-specific reads into three categories: paired-end reads, forward-only reads, and reverse-only reads. These reads are then processed separately through *DADA2* (Callahan et al. 2016) to denoise, dereplicate, merge paired reads, and remove chimeric sequences. This step returns ASV FASTA files and ASV count summary tables for four read types: merged paired-end reads, unmerged paired-end reads (filtered based on length and overlap criteria; Appendix 1.2), forward-only reads, and reverse-only reads. These files are the inputs for the *Anacapa Classifier* module for assigning taxonomy.

### **2.3 | Taxonomic assignment: *Anacapa Classifier* assigns taxonomy with *Bowtie2* and *BLCA***

The *Anacapa Classifier* module (Figure S1-1C; Appendix 1.3) assigns taxonomy to ASVs using *Bowtie2* and a modified version of *BLCA* (Gao et al. 2017). We verified that our modification to *BLCA* (namely, accepting *Bowtie2*-formatted SAM files rather than BLAST output files) does not influence taxonomic assignment (Appendix 4). In the first step of the *Anacapa Classifier*, *Bowtie2* queries ASVs against metabarcode-specific *CRUX* generated reference databases returning up to 100 alignments per ASV. The module uses *Bowtie2*'s "very-sensitive" preset to ensure high-quality alignments. The *Bowtie2* outputs are then processed with *Bowtie2-BLCA*, using multiple sequence alignment to probabilistically determine taxonomic identity by selecting the lowest common ancestor from the multiple weighted *Bowtie2* hits for each ASV. This module returns both detailed and brief reports of taxonomic assignment, and eight sets of taxonomy tables based on varying bootstrap confidence cutoffs (40-100).



### 3 | Benchmarking the *Anacapa Toolkit*

To benchmark the performance of the first three modules of the *Anacapa Toolkit*, we performed a series of quantitative tests of these modules on various metabarcodes and sequencing read types. Detailed methods and results from these comparisons are presented in Appendices 3 - 5. Briefly, to compare *CRUX*-generated databases to previously published reference databases, we conducted pairwise comparisons examining the total number of metabarcode specific sequences in the reference databases and the phylogenetic breadth of these databases for specific metabarcoding markers (Appendix 3). We found that *CRUX*-generated databases capture more metagenomic sequences and greater taxonomic diversity than published reference databases for three common metabarcoding loci: *COI*, *12S*, and Fungal *ITS* metabarcode loci.

To compare the performance of the *Anacapa Classifier*, we conducted Cross-Validation by Identity using published reference datasets and leave-one-out (10 fold) *COI* database comparisons following the methods of Edgar (2018) (see Appendix 4 for detailed methods and results). Results showed that the *Anacapa Classifier* consistently generated high-accuracy taxonomic assignments comparable to published classifiers. We also explored the consequences of varying bootstrap confidence cutoff on assigned taxonomy and found that the optimal value for the bootstrap confidence cutoff score varied across metabarcoding loci (Appendix 4). Finally, we verified that the *Anacapa Toolkit Quality Control and ASV Parsing* module and *Anacapa Classifier* module can both process longer (e.g. MiSeq) and shorter (e.g. HiSeq) DNA metabarcoding sequences (Appendix 5), expanding the utility of the *Anacapa* pipeline in comparison to existing methods.

#### 4| Case Study: Using the *Anacapa Toolkit* to assign taxonomy to field collected eDNA samples

To test the *Anacapa Toolkit* on field-collected eDNA metabarcoding datasets, we processed 30 seawater samples from kelp forests across the Southern California Channel Islands, including Anacapa Island. Seawater samples were amplified using *12S* (Miya et al. 2015) and *COI* (Leray et al. 2013) metabarcodes (see Appendix 6 for laboratory preparation and data analysis; Table S6.1). For the *12S* metabarcode, the *CRUX* module was critical for assigning taxonomy because there are no published reference databases for this locus that include the full breadth of amplifiable clades beyond fish taxa (Sato et al. 2018). Sequence data from these samples are available in NCBI (SRA accession SRP140860). Totaled across seawater samples, we generated 15,745,317 paired-end sequencing reads. Of these, 11,866,904 sequences reads were *12S* and 3,878,413 were *COI* resulting in 6,876 ASV and 6,287 ASVs, respectively, after filtering out singletons (Appendix 6). For both loci, we found that 99.5% were merged read pairs and <1% were unmerged paired, forward, or reverse only reads. The *Anacapa Toolkit*'s taxonomic assignments indicate that these ASVs matched to 21 eukaryotic phyla that could be further delimited within 49 classes, 295 families, and 414 genera and 533 species. Taxa identified included many of interest for natural resource managers including species of special concern (e.g. Basking shark, *Cetorhinus maximus*, and Giant black sea bass, *Stereolepis gigas*) and species that are the subject of focused monitoring efforts (California sheephead, *Semicossyphus pulcher*, and Ochre star, *Pisaster giganteus*) (Figure S1-2; Tables S6.2, S6.3). These results highlight the ability of eDNA to detect a wide breadth of marine life and its utility for biodiversity monitoring. A detailed and interactive summary of these seawater samples is available as the demo dataset of the *ranacapa* module (<https://gauravsk.shinyapps.io/ranacapa/>).

**Figure S1-2.** Taxonomic assignments from California environmental samples. Highlights the Anacapa Island kelp forest vertebrate families identified from the 12S metabarcodes. Families in bold are featured in the photographs.



## 5 | Conclusion

Biodiversity monitoring initiatives are increasingly using eDNA to inventory communities using multilocus metabarcoding. However, the lack of accurate and easily customizable bioinformatic pipelines is limiting the broader application of eDNA approaches. The *Anacapa Toolkit* provides enhanced functionality for eDNA projects and can be used for other common applications such as gut content analysis (Leray et al. 2013), autonomous reef monitoring structures (Ransome et al. 2017), and microbiomes (Bokulich et al. 2018).

Importantly, the *Anacapa Toolkit* is modular and its parameters are easily modifiable, making it easily adapted to user specific needs in several important ways. First, *CRUX* reference databases are compatible with alternative classifiers (Bokulich et al. 2018), and users can append their own reference sequences to *CRUX* databases as needed. Second, the *Quality Control and ASV Parsing* module is designed to process pooled metabarcoding libraries and automatically sort them by barcode and sample. The resulting output files (with the exception of unmerged paired reads) can be analyzed by most classifiers. Third, the *Anacapa Bowtie2-BLCA Classifier* can be applied to any high-throughput sequencing data, and process paired and unpaired reads. The robustness of *CRUX*-generated reference databases and the flexibility of the *Anacapa Toolkit* enables studies with a variety of metabarcoding loci to efficiently and transparently assign taxonomy, facilitating a diversity of eDNA approaches, ranging from basic ecology to biodiversity management and conservation.

## SUPPLEMENTAL CHAPTER 2

### **eDNA Metabarcoding bioassessment of endangered fairy shrimp (*Branchinecta* spp.)**

#### **Abstract**

Habitat degradation and land-use change severely threaten the survival of fairy shrimp and the vernal pools in which they live. Limiting effective fairy shrimp conservation management efforts is the ability to readily identify species without the capture and sacrifice of individuals for microscopy. Here we demonstrate that eDNA metabarcoding is an effective non-invasive tool for monitoring fairy shrimp. Results from ten Southern California vernal pools comparing eDNA and traditional dip-net methods showed that eDNA metabarcoding with *16S* rDNA provides exceptional species-level resolution. Importantly, while the two methods were concordant during early hydroperiods where adults were present, eDNA detected fairy shrimp for up to 2 months after dip-nets failed to detect any adults, expanding the time period for vernal pool monitoring. Together, these results provide resource managers a simple, cost-effective, and non-invasive method for biomonitoring endangered fairy shrimp species.

#### **Introduction**

Fairy shrimp (Crustacea: Malacostraca: Anostraca) occur in ephemeral vernal pool habitats worldwide providing critical ecosystem function as the primary food source for migratory birds and other aquatic species (Keeley and Zedler 1998, Zedler 2003). However, fairy shrimp are increasingly threatened as fragile vernal pool ecosystems are lost to habitat

degradation from urban development and agriculture (Bauder and McMillan 1998, King 1998, Zacharias and Zamparas 2010, Simovich et al. 2013).

Vernal pool loss is pronounced in highly modified ecosystems such as California where 95% of original coastal vernal pool habitat has been lost (Bauder and McMillan 1998). As a result, in California alone, four *Branchinecta* fairy shrimp species are listed under the Endangered Species Act. Three Endangered species (*Branchinecta conservatio* (Eng et al. 1990), *B. longiantenna* (Eng et al. 1990), and *B. sandiegonensis* (Fugate 1993)) and one Threatened species (*B. lynchi* (Eng et al. 1990)) are actively managed for their conservation (Eng et al. 1990, Fugate 1993, Interior 1997, Bauder and McMillan 1998, Simovich et al. 2013).

Effective management of fairy shrimp requires the ability to swiftly identify species, a multifaceted challenge. First, reliable identification of *Branchinecta* sp. is time and labor intensive, requiring extensive morphological examination of sexually mature specimens through microscopy (Eriksen and Belk 1999, Rogers 2002). Second, the window for vernal pool collection efforts is unpredictable given precipitation patterns (Eng et al. 1990). Third, morphological identification is destructive, requiring sacrifice of individuals that further reduces endangered fairy shrimp populations. These challenges are exemplified in Southern California where the generalist shrimp *B. lindahli* (Packard 1883) is expanding into vernal pool systems previously known to only harbor *B. sandiegonensis*, outcompeting this endangered species where they co-occur (Simovich et al. 2013). Conservation of the endangered *B. sandiegonensis* requires managing *B. lindahli*, but morphological identification of these species is challenging (Patel et al. 2018), highlighting a need for novel alternative approaches.

Environmental DNA (eDNA) uses DNA barcoding or metabarcoding to identify species by isolating and sequencing freely associated DNA organisms leave in their environment (Deiner

et al. 2017). This approach has shown versatility in a variety of aquatic environments (Senapati et al. 2018), detecting amphibian, bird, and crustacean taxa, including rare and difficult-to-sample species (Deiner et al. 2017, Senapati et al. 2018). eDNA is well-suited to detecting organisms that are difficult to identify morphologically, given the relative ease of sample collection and DNA barcoding (Deiner et al. 2017, Curd et al. 2019). eDNA is also non-invasive, avoiding sacrifice of target organisms (Deiner et al. 2017). These features make eDNA an attractive tool for monitoring vernal pool ecosystems, particularly fairy shrimp.

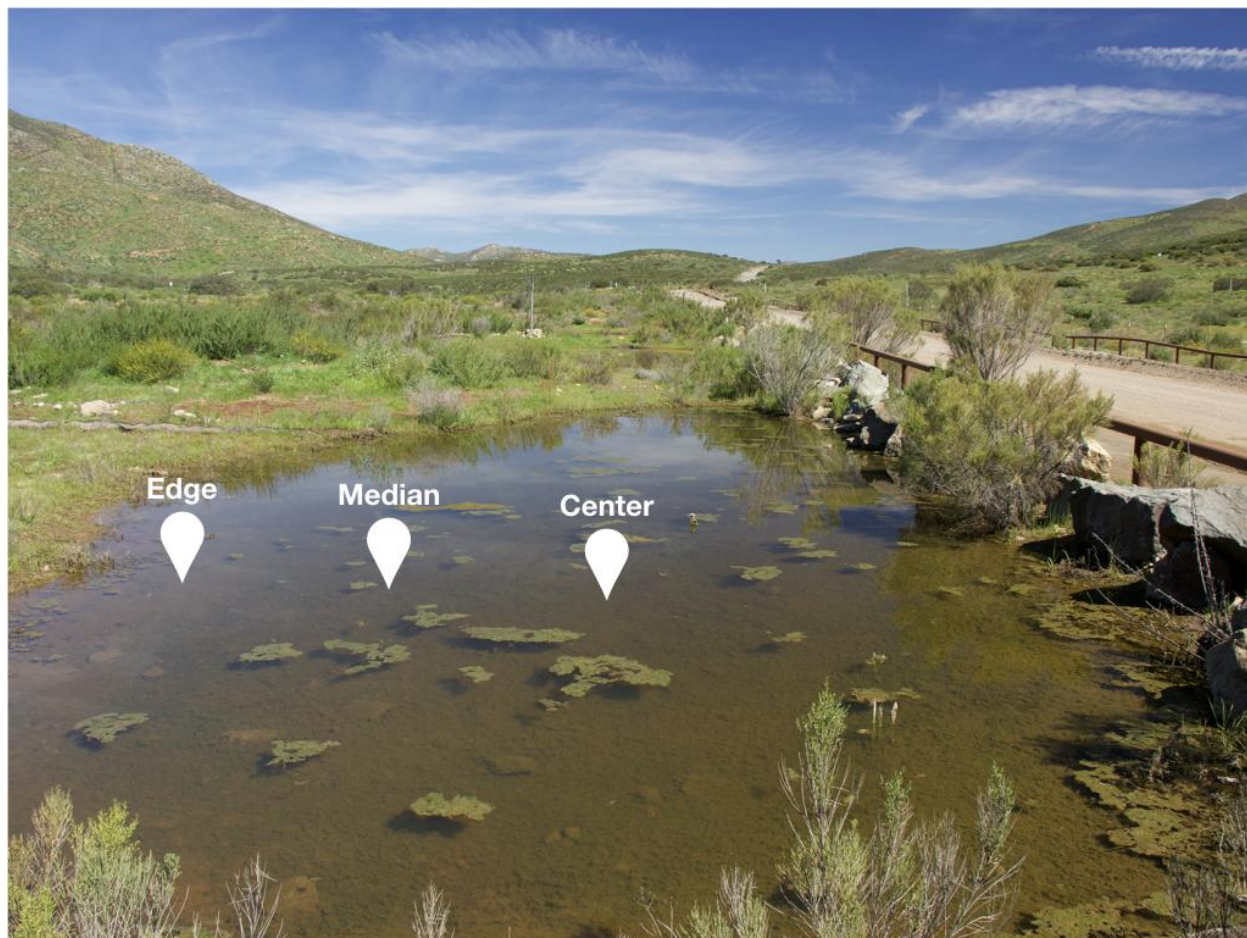
Here, we develop an eDNA metabarcoding method for vernal pool water samples and compare our results with traditional dip-net sampling of *B. sandiegonensis* and *B. lindahli*. We then test the assay across pools representing a range of potential habitat for both fairy shrimp species.

## Methods

### *Study Sites*

We conducted eDNA and dip-net surveys at 10 vernal pools known to previously contain either *B. sandiegonensis* or *B. lindahli* in San Diego County, California, USA (The Chapparal Lands Conservancy 2017a) (Figure S2-1). Eight pools (VP 1-8) were reported to exclusively contain *B. sandiegonensis* but with high potential to harbor *B. lindahli*. VP-9 was known to only have *B. lindahli* (The Chapparal Lands Conservancy 2017b). VP-10 is known for its abundance of purely *B. sandiegonensis*.

**Figure S2-1.** Vernal Pool Sampling Stations. Photograph of a vernal pool in the Proctor Valley Vernal Pool Habitat Restoration Project site. The three locations of eDNA sample collection are labeled center, median, and edge, here in referred to as replicates.



We sampled most vernal pools three times across the hydroperiod to provide data across the fairy shrimp life cycle (Hildrew 1985). We subsequently refer to these sampling events as Time-1 (January 23, 2017), Time-2 (February 13, 2017), and Time-3 (March 7, 2017). We only sampled VP-9 at Time-3 when notified it had adult *B. lindahli* present. VP-8 was inaccessible at Time-2.



### *eDNA Field Collection*

As false positives may result from contamination during eDNA collection, extraction, and amplification, we employed multiple steps to minimize contamination (See Supplemental Methods) (Goldberg et al. 2016).

At vernal pools VP 1-8 and VP-10 we collected three 1L replicate water samples at the center, median, and edge to account for potential spatial differences in eDNA signatures (Figure S2-1, Supplemental Table S2-S11). At VP-9, we only sampled 1L due to the pools' small size (<1m diameter). We used a 240µm net to exclude all animals from entering the Nalgene bottle, returning captured organisms to their pool.

We isolated eDNA using a 0.22 µm Sterivex filter cartridge and either gravity filtration or a 50 mL syringe (Curd et al. 2019). We then transferred the filter cartridge on ice to the lab where they were stored at -20 °C until extracted. Due to turbidity, we filtered samples until the cartridge clogged. Sample volume ranged from 50 ml to 450 ml reported in Supplemental Table S2-S1.

### *Library Preparation*

We amplified samples using a *16S* rDNA metabarcode (Kelly et al. 2016) following the methods of Curd et al. (2019) (see Appendix – D Supplemental Methods for detailed laboratory preparation). We chose this primer because preliminary analyses using *COI* metabarcodes failed to resolve *Branchinecta* to species level (Data not shown). Briefly, we performed amplification in triplicate following the touchdown PCR protocol of Curd et al. (2019). We then performed a second indexing PCR using Illumina Nextera Indexes. Libraries were then prepared following Curd et al. (2019) and sequenced on two separate MiSeq runs with Reagent Kit V3.

## Bioinformatics

We processed the resulting sequence libraries separately using the *Anacapa Toolkit* (Curd et al. 2019) (See Appendix – D Supplemental Methods). We used the *Anacapa Toolkit CRUX*-generated *16S* rDNA reference database built February 2019 (Curd et al. 2019), supplemented with the above *Branchinecta* barcode sequences.

We followed the decontamination protocol of Kelly et al. (2018). We ran site-occupancy modeling to discriminate between potential PCR artifacts and true species detections following Chambert et al. (2018) (See Appendix – D Supplemental Methods). We then observed true positive and false positive presence and absence rates of *B. sandiegonensis* and *B. lindahli* across all pools.

## Reference DNA Barcodes

To ensure our reference library included target species, we generated DNA sequences of 10 *B. sandiegonensis* individuals and 9 *B. lindahli* individuals confirmed through morphological analysis. All reference barcodes were accessioned into GenBank (Supplemental Table S2-S2).

## Traditional Dip-net Surveys

We simultaneously conducted traditional dip-net surveys during eDNA sampling to compare the effectiveness of each method. All adult *Branchinecta* specimens were morphologically sorted to species level (Supplemental Table S2-S3). We then calculated site occupancy rates of dip-net samples at the landscape level according to Chambert *et al.* 2018 (See Appendix – D Supplemental Methods).

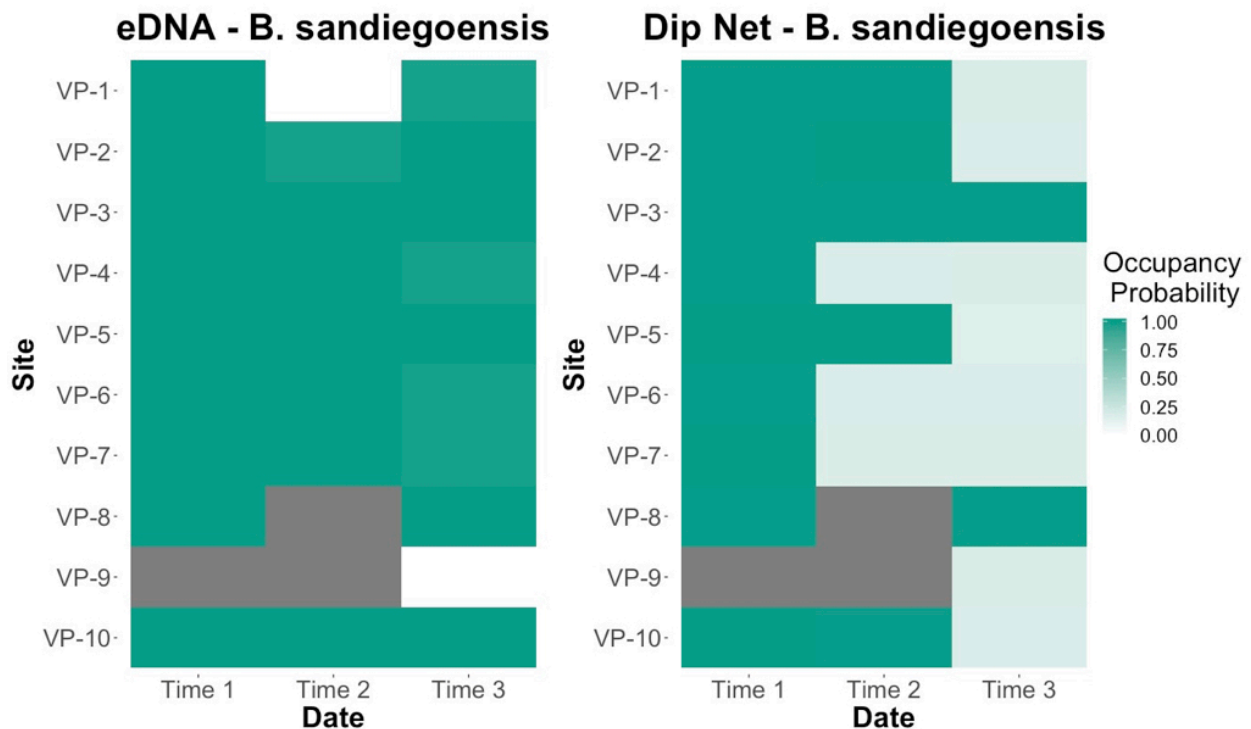
## Results

We generated 7.1 million sequences from 79 eDNA samples from 10 vernal pools spanning three sampling periods, detecting a total of 2630 amplicon sequence variants (ASVs) (Supplemental Table S2-S4). Sanger sequencing of 10 *B. sandiegonensis* and 9 *B. lindahli* (Supplemental Table S2-S2) confirmed diagnostic SNPs within the target locus, distinguishing four *Branchinecta* species (*B. sandiegonensis*, *B. lindahli*, *B. lynchi*, and *B. paludosa*), allowing us to discriminate between *B. sandiegonensis* and *B. lindahli* through eDNA metabarcoding.

The *Anacapa Toolkit* assigned 201 ASVs to *B. sandiegonensis*, 9 ASVs to *B. lindahli*, and 21 ASVs to *Branchinecta sp.* (Supplemental Table S2-S5 and S2-S6), detecting *Branchinecta sp.* in 26 of 27 (96.3%) sampled vernal pools across all three time points. Results confirmed *B. sandiegonensis* in 25 of 27 pools (92.6%). In contrast, eDNA detected *B. lindahli* in VP-9 at Time-3, its only sampling period, as well as in a single replicate each in pools VP-1 and VP-5, only at Time-1. eDNA failed to detect *Branchinecta sp.* in one sample, VP-1 at Time-2, despite recovering other invertebrate diversity (Supplemental Table S2-S4).

Dip-net surveys detected *B. sandiegonensis* in 16 out of 27 (59.3%) total sampling events. Dip-net surveys detected *B. sandiegonensis* predominantly in Time-1 (10 of 10 pools, 100%) and Time-2 (5 of 8 pools, 62.5%). However, by Time-3, dip-net surveys only detected *B. sandiegonensis* in 2 of 10 (20%) pools sampled (Figure S2-2). Dip-net surveys detected *B. lindahli* in VP-9 at Time-3, its only sampling period.

**Figure S2-2.** Change in Detection Probability of *Branchinecta sandiegonensis* for eDNA and dip-net surveys. Shade of green denotes occupancy rate of *B. sandiegonensis* across three sampling time points (Time-1, Time-2, and Time-3), highlighting higher performance of eDNA compared to dip-net sampling. Grey indicates pools and times where sampling did not occur.



Of the 16 pools where dip-net surveys recovered *B. sandiegonensis*, eDNA detected *B. sandiegonensis* in 15 pools (93.8%) (Figure S2-2), failing to detect *B. sandiegonensis* in VP-1 during Time-2. Importantly, eDNA detected *B. sandiegonensis* in 10 additional vernal pool sampling events in which contemporaneous dip-net surveys did not detect *Branchinecta*, despite detection by dip-net earlier in the hydrological period. Both eDNA and dip-net surveys recovered *B. lindahli* in the one VP-9 sample. Results from site occupancy modeling indicate eDNA

metabarcoding had higher sensitivity and specificity for detecting *B. sandiegonensis* than dip-net surveys (Table S2-1), but dip-net surveys had higher sensitivity and specificity for detecting *B. lindahli* (Table S2-1).

**Table S2-1. Site Occupancy Modeling Estimated Sensitivity and Specificity of eDNA metabarcoding and dip-net surveys**

Estimates of sensitivity and specificity of eDNA and dip-net sampling methods for *B. sandiegonensis* and *B. lindahli* based on Chambert et al. 2018. 95% confidence intervals are reported in parentheses.

Survey	Species	True Positive Rate	False Positive Rate	True Negative Rate	False Negative Rate	Sensitivity	Specificity
eDNA	<i>B. sandiegonensis</i>	98% (95-100%)	1% (0-4%)	99% (96-100%)	2% (1-5%)	98.9%	99.0%
eDNA	<i>B. lindahli</i>	66% (31-97%)	1% (0-2%)	99% (98-100%)	34% (3-69%)	98.5%	74.4%
Dip-net	<i>B. sandiegonensis</i>	88% (68-99%)	1% (0-3%)	99% (97-100%)	12% (1-32%)	98.8%	89.2%
Dip-net	<i>B. lindahli</i>	81% (46-100%)	1% (0-3%)	99% (97-100%)	19% (0-54%)	98.8%	83.9%

## Conclusions

This study demonstrates the power of eDNA to detect both endangered (*B. sandiegonensis*) and common (*B. lindahli*) fairy shrimp in seasonal vernal pool ecosystems of Southern California, adding to a growing body of literature on the utility of eDNA methods (Thomsen and Willerslev 2014, Deiner et al. 2017). Results demonstrate strong concordance between traditional dip-net and eDNA metabarcoding surveys as found in previous eDNA

ground truthing studies (Thomsen and Willerslev 2014, Deiner et al. 2017). Importantly, eDNA methods had higher specificity and sensitivity than dip-net methods for the detection of the endangered *B. sandiegonensis*. Although we found eDNA had lower specificity and sensitivity for *B. lindahli*, this is likely a statistical artifact of site occupancy modelling on a single pool with *B. lindahli*. Overall, these results demonstrated eDNA is an effective way to non-destructively survey vernal pools for fairy shrimp.

While eDNA and dip-net surveys revealed congruent results for *B. sandiegonensis*, there were minor discrepancies. Specifically, eDNA failed to detect *B. sandiegonensis* in VP-1 at Time-2 in which dip-net sampling confirmed its presence. Interestingly, eDNA detected *B. sandiegonensis* at this pool during Time-1 and Time-3. This anomalous absence likely results from decontamination procedures that removed corresponding *B. sandiegonensis* sequences because of low presence in field and PCR blanks (137 max reads per blank, 514 total reads across all controls). Despite sterile procedures, low, but persistent, levels of contamination is a well-known problem within eDNA studies necessitating exacting decontamination methods (Goldberg et al. 2016, Davis et al. 2018), which likely resulted in this false negative.

eDNA metabarcoding detected fairy shrimp in 10 sampling events in which they were not detected using dip-net surveys. Detection of *B. sandiegonensis* by eDNA occurred in pools in which dip-nets had confirmed its presence 2 months earlier, suggesting *B. sandiegonensis* eDNA can persist past the presence of adults, perhaps as eggs, as the known degradation of aquatic eDNA occurs on the scale of hours to days (Deiner et al. 2017, Senapati et al. 2018). This finding is important as the ability of eDNA to detect endangered *Branchinecta* in the absence of reproductive adult stages allows monitoring to occur anytime during the hydroperiod, and potentially during the dry season as eDNA is recoverable from sediments (Deiner et al. 2017).

These detections were unlikely to be false-positives, given our calculated error rates (Table S2-1).

Although we found that eDNA had less sensitivity and specificity for *B. lindahli* detection, eDNA detected *B. lindahli* in the one pool where dip-net surveys detected it as well as two additional vernal pools where adults were not observed. *B. lindahli* is routinely reported in vernal pools less than 10m across a dirt road (The Chapparal Lands Conservancy 2017a), and may have been introduced into the pools where detected by eDNA. Alternatively, these detections could be sequencing artifacts. In either case, these results warrant further investigation and demonstrate the potential early warning capabilities of eDNA methods.

While our results highlight the utility of eDNA in monitoring *Branchinecta*, an important caveat is that *B. sandiegonensis* and *B. lindahli* can purportedly hybridize (Patel 2018). While hybridization can be excluded in our study as both species were only found together in two pools and one time point, future work should focus on advancing population genomic eDNA approaches to identify hybridized *Branchinecta* species.

Ultimately, our results demonstrate eDNA metabarcoding is an effective, sensitive, and non-invasive method that provides improved biomonitoring of fairy shrimp, and represents the best available science for monitoring these endangered species. The sensitivity, specificity, and cost-effectiveness of eDNA metabarcoding approaches should allow for the dramatic expansion of vernal pool monitoring, providing resource agencies critical information for vernal pool management and restoration efforts.

## APPENDIX A – CHAPTER 1

### **Supplemental Methods**

#### *eDNA Filter Extraction*

In the laboratory, we extracted eDNA from filter cartridges within one week of collection using a modified DNeasy Tissue and Blood Qiagen Kit, adding the proteinase K and ATL buffer reagent the sterile filter cartridge (Spens et al. 2017, Curd et al. 2019). We then stored extracted DNA at -20°C before PCR.

#### *Contamination Precautions*

As false positives may result from contamination during eDNA collection as well as during DNA extraction and amplification, we employed multiple steps to minimize contamination and generation of false positives. Prior to water sample collection in the field, we sterilized all containers, supplies, and work surfaces with at least 10% bleach solution and always wore gloves to minimize the risk of contamination. In addition, we performed all DNA extractions in a PCR-free laboratory area, and all PCR preparations in an AirClean 600 PCR Workstation (AirClean Systems, Creedmoor, NC, USA) located in a clean room at UCLA dedicated to PCR/qPCR preparations. Prior to use, we sterilized the AirClean 600 PCR Workstation and pipettes before and after use with 10-30% bleach followed by a 30 min ultraviolet light (UV) treatment. Filtered pipette tips were used for all pre-PCR protocols. Lastly, to test for any possible contamination, we implemented negative controls at each step, including field collections, DNA extraction, and PCR.



### *Library Preparation*

We amplified approximately 176 bp of mitochondrial *12S* rDNA, using two fish primer sets: MiFish Universal and MiFish Elasmobranch (Miya et al. 2015) modified using Nextera adapter (Illumina, San Diego, CA, USA) sequences (Curd et al. 2019). We performed PCR amplification in triplicate using a 25  $\mu$ L reaction mixture containing 12.5  $\mu$ L QIAGEN Multiplex Taq PCR 2x Master Mix, 6.5  $\mu$ L of dH<sub>2</sub>O, 2.5  $\mu$ L of each primer (2  $\mu$ mol/L), and 1  $\mu$ L template DNA. Thermocycler parameters employed a touchdown program: initial denaturation at 95°C for 15 min, 13 cycles of denaturation at 94°C for 30 sec, beginning annealing at 69.5°C for 39 sec (temperature was decreased by 1.5°C every cycle until 50°C was reached), and extension at 72°C for 1 min. Thirty five additional cycles were carried out at an annealing temperature of 50°C, followed by a final extension at 72°C for 10 min. For negative PCR controls, we used molecular grade water in place of DNA extractions. To confirm successful PCRs and product size, we electrophoresed all PCR products on 2% agarose gels.

We pooled triplicate PCR reactions using 5 $\mu$ L volume from each PCR, and then pooled PCR samples were cleaned using Serapure magnetic bead protocol (Faircloth and Glenn 2014). We quantified bead-cleaned samples with the Quant-iT™ broad range dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) on a Victor3 plate reader (Perkin Elmer Waltham, MA, USA). We indexed the sample libraries using unique combinations of the Nextera Index A and D Kit (Illumina, San Diego, CA, USA) and KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Sigma Aldrich, St. Louis, MO, USA) (Curd et al. 2019). Indexing was performed with a second PCR using a 25  $\mu$ L reaction mixture containing 12.5  $\mu$ L of Kapa HiFi HotStart Ready mix, 0.625  $\mu$ L of primer i7, 0.625  $\mu$ L of primer i5, and 10 ng of template DNA. Index thermocycling parameters were denaturation at 95°C for 5 min, 5 cycles of denaturation at 98°C

for 20 sec, annealing at 56°C for 30 sec, extension at 72°C for 3 min, followed by a final extension at 72°C for 5 min. To confirm successful PCR and correct product size, we electrophoresed PCR products on 2% agarose gels. We then bead cleaned and quantified DNA concentration, as described above so that we could pool samples so as to have equal copy number. We then sequenced the library on a MiSeq using Reagent Kit V3 with 20% PhiX added. Sequencing was conducted at Technology Center for Genomics & Bioinformatics (University of California–Los Angeles, CA, USA).

### *Bioinformatics*

We processed the resulting sequence library using the *Anacapa Toolkit*, which performs quality control, amplicon sequence variant (ASV) parsing, and taxonomic assignment using user generated custom reference databases (Curd et al. 2019). We employed default parameters except for using a 70% minimum length and 70% mismatch for Bayesian Lowest Common Ancestor taxonomic classification.

## APPENDIX B – CHAPTER 2

### Supplemental Methods

#### *First PCR Protocol*

We performed PCR amplification in triplicate using a 25  $\mu$ L reaction volume containing 12.5  $\mu$ L QIAGEN Multiplex Taq PCR 2x Master Mix (Qiagen Inc., Valencia, CA, USA), 6.5  $\mu$ L of dH<sub>2</sub>O, 2.5  $\mu$ L of each primer (2  $\mu$ mol/L), and 1  $\mu$ L DNA extraction. PCR thermocycling employed a touchdown profile with an initial denaturation at 95°C for 15 min to activate the DNA polymerase. This was followed by 13 cycles of a denaturation step at 94°C for 30 sec, an annealing step starting at 69.5°C for 30 sec which was then decreased by 1.5°C for each cycle (last cycle was 50°C), and an extension step at 72°C for 1 min. This was followed by 35 additional cycles carried out at an annealing temperature of 50°C using the same denaturation and extension steps above, and ending with a final extension at 72°C for 10 min (Curd et al. 2019).

#### *Second Indexing PCR Protocol*

This second indexing PCR was performed using a 25  $\mu$ L reaction mixture containing 12.5  $\mu$ L of Kapa HiFi HotStart Ready mix, 0.625  $\mu$ L of primer i7, 0.625  $\mu$ L of primer i5, and 10ng of template DNA, and used the following thermocycling parameters: denaturation at 95°C for 5 min, 5 cycles of denaturation at 98°C for 20 sec, annealing at 56°C for 30 sec, extension at 72°C for 3 min, followed by a final extension at 72°C for 5 min (Curd et al. 2019).

## *Decontamination*

### Estimation of Index Hopping

All samples were pooled into a final library and sequenced on a single MiSeq run. Each sample is identified by two sets of molecular barcodes in a unique combination. However, recent evidence has found that there is the potential for indexes to hop from one molecular to another, leading to the incorrect sample assignment during demultiplexing (Costello et al. 2018). To estimate the frequency of index hopping we included a positive control of a non-native fish taxa which we know will not be found in our eDNA samples (Kelly et al. 2018). Index hopping will lead to environmental sequences occurring in the positive control and vice versa. To estimate the frequency of index hopping we modeled the composition of environmental sequences observed on the positive controls and subtract these sequences from the environmental samples run. For example, if 12 reads of Garibaldi (*Hypsypops rubicundus*) are found in the positive control, we subtract 12 reads from the read counts of Garibaldi found in all environmental samples.

### Remove Contamination from Negative Controls

Here we remove ASVs that occur in positive and negative controls with higher proportions than environmental samples using *R* package *microDecon* (McKnight et al. 2019). We used the standard parameters and grouped samples by location.

### Site Occupancy Modelling

The goal of site occupancy modeling is to determine whether the presence of an ASV is a true reflection of biological reality (i.e. the fish was present) or the result of a PCR artifact. This is challenging because it requires discriminating between PCR artifacts from rare but real organisms. Site occupancy modeling provides a robust statistical framework to determine if the

presence pattern of an ASV reflects a PCR artifact or rare organism (Royle & Link, 2006; Schmidt et al., Kery, Ursenbacher, Hyman, & Collins, 2013). As with all site occupancy models, we assume occupancy state does not change between sampling efforts and that detections at a site are completely independent (Lahoz-Monfort et al. 2016). Whether a site is occupied ( $z=1$ ) or not ( $z=0$ ) can be evaluated using a Bernoulli trial with probability of occupancy ( $z \sim \text{Bern}(w)$ ). The occupancy probability is constant within a site, which is incorporated in the model through a logit-linear model. The binomial parameter of conditional on site occupancy status was defined as  $P[i] \leftarrow z[i] \times P11 + (1-z[i]) \times P10$  where the probability of species occurrences at a location (denoted PSI) the conditional probability of species occurrence within an eDNA sample from a site given that the species was truly at the site (true positive detection) (denoted PS11) the conditional probability of a species occurrence within an eDNA sample from a site given that the species was falsely at the site (false positive detection) (denoted PS10). The probability occurrence function used was the following:

$$\text{Probability of Occurrence} = \frac{(\text{PSI} \times (P11^N) \times (1-P11)^{(K-N)})}{((\text{PSI} \times (P11^N) \times (1-P11)^{(K-N)}) + ((1-\text{PSI}) \times (P10^N) \times ((1-P10)^{(K-N)})))}$$

Where K is the number of samples taken within a site and N is the number of ASV detections within a site.

Occupancy probability (PSI) was modeled with priors from a left-skewed beta distribution  $\alpha = 1$  and  $\beta = 6$  as we assume most species are rare in most locations. We modeled the true positive probability (P11) with left-skewed beta distribution  $\alpha = 6$  and  $\beta = 1$  as we assume our primers to a reasonably good job of detecting species if a species is present. False positive probability (P10) was modeled with priors from a left-skewed beta distribution  $\alpha = 1$

and  $\beta = 30$  as we assume the false positive rate of detection is unlikely to approach the true positive rate.

For each ASV we created a presence-absence matrix for each site to feed the model. Each matrix was 3 sites by 9 samples. The occurrence of a sequence for one ASV in a given replicate water sample was treated as a detection with that site. Each pattern of occurrence for a given ASV within a given site was considered a case. We then summarized the number of occurrences of each case and ran each case through a separate occupancy model to reduce computational time. Each unique model was run 10 times in order to filter out cases in which the model converged into a local maxima. We then removed all ASVs which had a modeled site occupancy probability of less than 75%.

#### Calculating eDNA Index Scores

The eDNA index was computed following the methods of Kelly et al. (2019). This was accomplished by first calculating the mean read count for each assigned taxonomy and then calculating the relative abundance of each ASV; number of reads of each ASV divided by the total number of reads per sample. The relative abundance of each taxa in each sample was then divided by the maximum abundance for a given species across all samples to generate the eDNA index. The index thus normalizes the read count per species and per sample. The eDNA index values 0 to 1 for each taxa, allowing for abundance comparisons of a specific taxa across sites.

#### *Visual Transect Surveys*

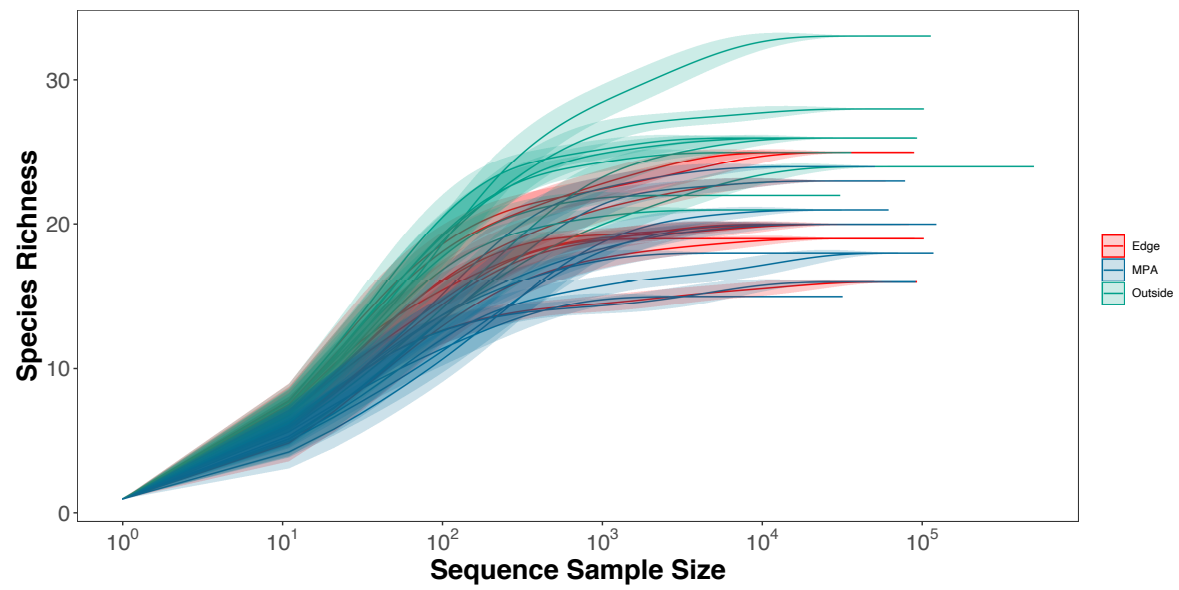
The four visual fish transects record all indicator fish species within a 3 m tall x 2 m wide x 50 m long area. The roving diver fish count is conducted by having divers gradually swim 10 m on both sides of a 100 m transect line for 30 minutes counting and sizing all fish species. Lastly, the

four video-taped transects are conducted across a 50 m transect. Together these data provide robust information on the diversity and size frequency of fish species at a given site (Sprague et al. 2013).

## Supplemental Results

The unique taxa found at the outside site were sand basses (*Paralabrax* sp.), kelpfishes (*Gibbonsia* sp.), CA clingfish (*Gobiesox rhesodon* and *Rimicola muscarum*), Horn shark (*Heterodontus francisci*), Yellowtail amberjack (*Seriola lalandi*), Rainbow scorpionfish (*Scorpaenodes xyris*), Reef finspot (*Paraclinus integripinnis*), and eels (Anguilliformes and *Facciolella gilberti*) (Supplemental Table 2-S4). The unique species found at the edge site were the Island kelpfish (*Alloclinus holderi*) and the Roughback sculpin (*Chitonotus pugetensis*) (Supplemental Table 2-S4). The species shared between the two sites outside the MPA were Painted greenling (*Oxylebius pictus*), Blackeyed goby (*Rhinogobiops nicholsii*), Fine-scaled triggerfish (*Balistes polylepis*), Speckled sanddab (*Citharichthys stigmaeus*), Mussel blenny (*Hypsoblennius jenkinsi*), and Crevice kelpfish (*Gibbonsia montereyensis*) (Supplemental Table 2-S4). The unique species found at the MPA site included the California scorpionfish (*Scorpaena guttata*), the Orangethroat pikeblenny (*Chaenopsis alepidota*), the Sarcastic fringehead (*Neoclinus blanchardi*), Spotted cusk eel (*Chilara taylori*), and the California lizardfish (*Synodus lucioceps*). The edge and MPA sites shared one species: the Fantail flounder (*Xystreurus liolepis*). The outside and MPA sites shared three species kelp perch (*Brachyistius frenatus*), Rubberlip surfperch (*Rhacochilus toxotes*), the Roughcheek sculpin (*Ruscarius creaseri*), and the C-O sole (*Pleuronichthys coenosus*) (Supplemental Table 2-S4).

**Supplemental Figure 2-S1:** Species Richness Sequence Depth Rarefaction





## APPENDIX C – CHAPTER 3

### Supplemental Methods

#### Estimation of Index Hopping

Technical replicates of each barcode were pooled into a six libraries and each separately sequenced on a single NextSeq run. Each sample is identified by two sets of unique molecular barcodes. However, recent evidence has found that there is the potential for indexes to hop from one molecular to another, leading to the incorrect sample assignment during demultiplexing (Costello et al. 2018). To estimate the frequency of index hopping we included two positive controls of a non-native vertebrate taxa which we know will not be found in our eDNA samples (Kelly et al. 2018). Index hopping will lead to environmental sequences occurring in the positive control and vice versa. To estimate the frequency of index hopping we modeled the composition of environmental sequences observed on the positive controls and subtract these sequences from the environmental samples run. For example, if 12 reads of Garibaldi (*Hypsypops rubicundus*) are found in the positive control, we subtract 12 reads from the read counts of Garibaldi found in all environmental samples.

#### Remove Contamination from Negative Controls

Here we remove ASVs that occur in positive and negative controls with higher proportions than environmental samples using *R* package *microDecon* (McKnight et al. 2019). We used the standard parameters and grouped samples by site (including all technical and biological replicates).

## Site Occupancy Modelling

The goal of site occupancy modeling is to determine whether the presence of an ASV is a true reflection of biological reality (i.e. the fish was present) or the result of a PCR artifact. This is challenging because it requires discriminating between PCR artifacts from rare, but real organisms. Site occupancy modeling provides a robust statistical framework to determine if the presence pattern of an ASV reflects a PCR artifact or rare organism (Royle & Link, 2006; Schmidt et al., Kery, Ursenbacher, Hyman, & Collins, 2013). As with all site occupancy models, we assume occupancy state does not change between sampling efforts and that detections at a site are completely independent (Lahoz-Monfort et al. 2016). Whether a site is occupied ( $z=1$ ) or not ( $z=0$ ) can be evaluated using a Bernoulli trial with probability of occupancy ( $z \sim \text{Bern}(w)$ ). The occupancy probability is constant within a site, which is incorporated in the model through a logit-linear model. The binomial parameter of conditional on site occupancy status was defined as  $P[i] \leftarrow z[i] \times P11 + (1-z[i]) \times P10$  where the probability of species occurrences at a location (denoted PSI) the conditional probability of species occurrence within an eDNA sample from a site given that the species was truly at the site (true positive detection) (denoted PS11) the conditional probability of a species occurrence within an eDNA sample from a site given that the species was falsely at the site (false positive detection) (denoted PS10). The probability occurrence function used was the following:

$$\text{Probability of Occurrence} = \frac{(\text{PSI} \times (P11^N) \times (1-P11)^{(K-N)})}{((\text{PSI} \times (P11^N) \times (1-P11)^{(K-N)}) + ((1-\text{PSI}) \times (P10^N) \times ((1-P10)^{(K-N)}))}, \quad (1)$$

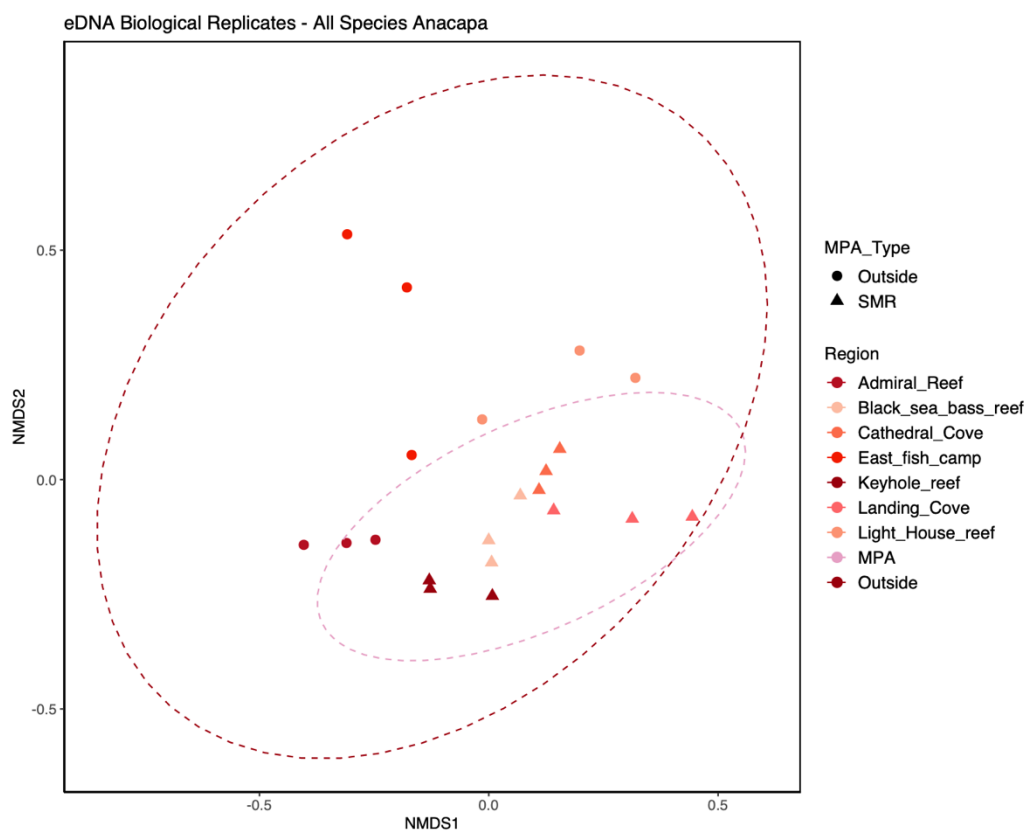
Where  $K$  is the number of samples taken within a site and  $N$  is the number of ASV detections within a site.

We used sensibly informative priors for parameter estimations. First, we assume that our primers do a reasonably good job of detecting species if the species are present (Chambert et al. 2018, Doi et al. 2019). Thus, true positive probability ( $P_{11}$ ) and occupancy probability ( $\Psi$ ) were modeled with priors from a left-skewed beta distribution where  $\alpha = 2$  and  $\beta = 2$ . We also assumed that the false-positive rate of detection is unlikely to approach the true-positive rate. Thus, false positive probability ( $P_{10}$ ) was modeled with priors from a left-skewed beta distribution where  $\alpha = 1$  and  $\beta = 10$ . *Stan* occupancy models are included in Supplemental Materials as attached text files.

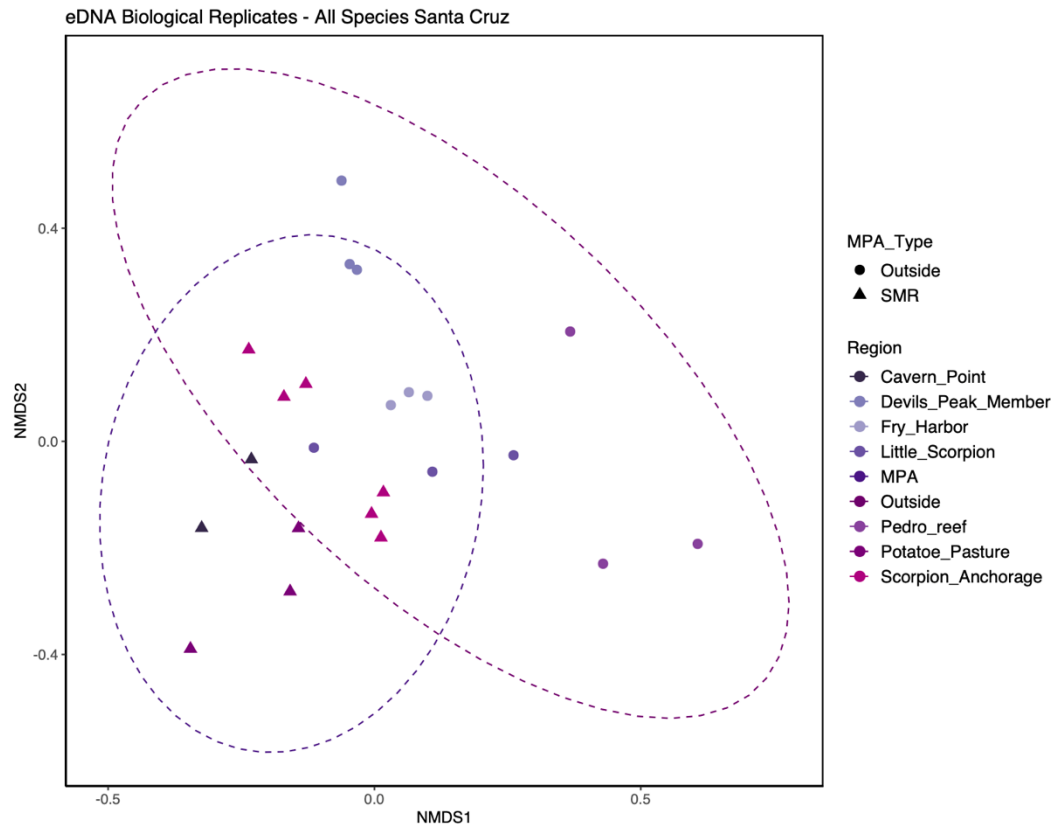
For each ASV we created a presence-absence matrix for each site to feed the model. Each matrix was 44 sites by 9 samples. The occurrence of a sequence for one ASV in a given technical replicate water sample was treated as a detection with that site. Each pattern of occurrence for a given ASV within a given site within a given biological replicate was considered a case. We then summarized the number of occurrences of each case and ran each case through a separate occupancy model to reduce computational time. Each unique model was run 10 times in order to filter out cases in which the model converged into a local maxima. We then removed all ASVs which had a modeled site occupancy probability of less than 65%.

## Supplemental Figures

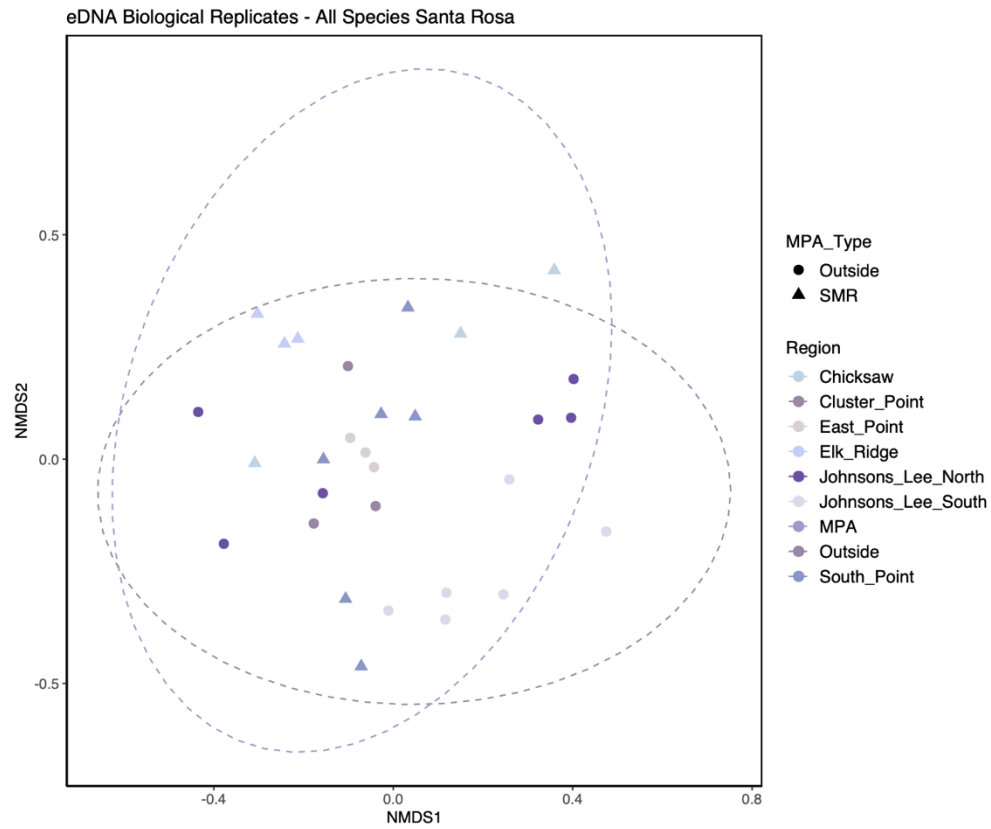
**Supplemental Figure 3-S1.** NMDS of Bray-Curtis Dissimilarities between eDNA Biological Replicates at Anacapa Island Using All Species Observed by eDNA. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.124).



**Supplemental Figure 3-S2.** NMDS of Bray-Curtis Dissimilarities between eDNA Biological Replicates at Santa Cruz Island Using All Species Observed by eDNA. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.093).

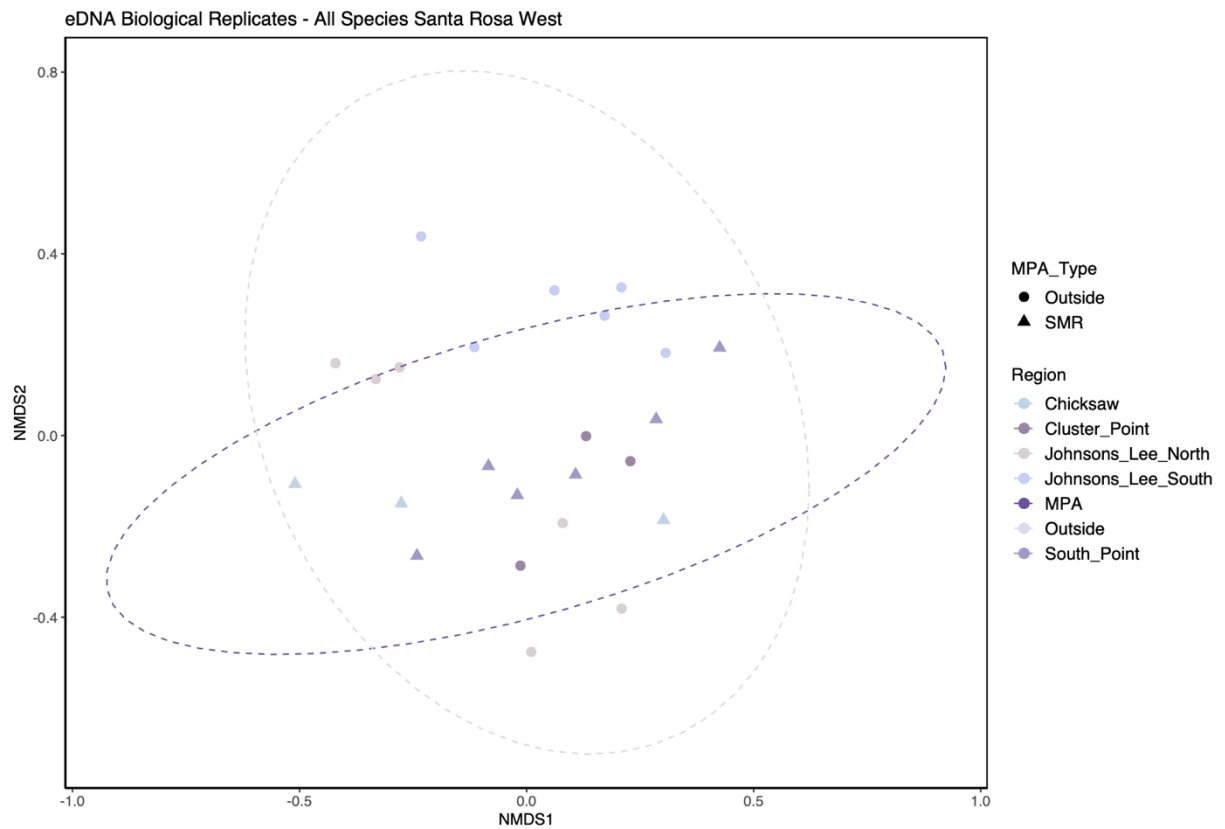


**Supplemental Figure 3-S3.** NMDS of Bray-Curtis Dissimilarities between eDNA Biological Replicates at Santa Rosa Island Using All Species Observed by eDNA. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.211).

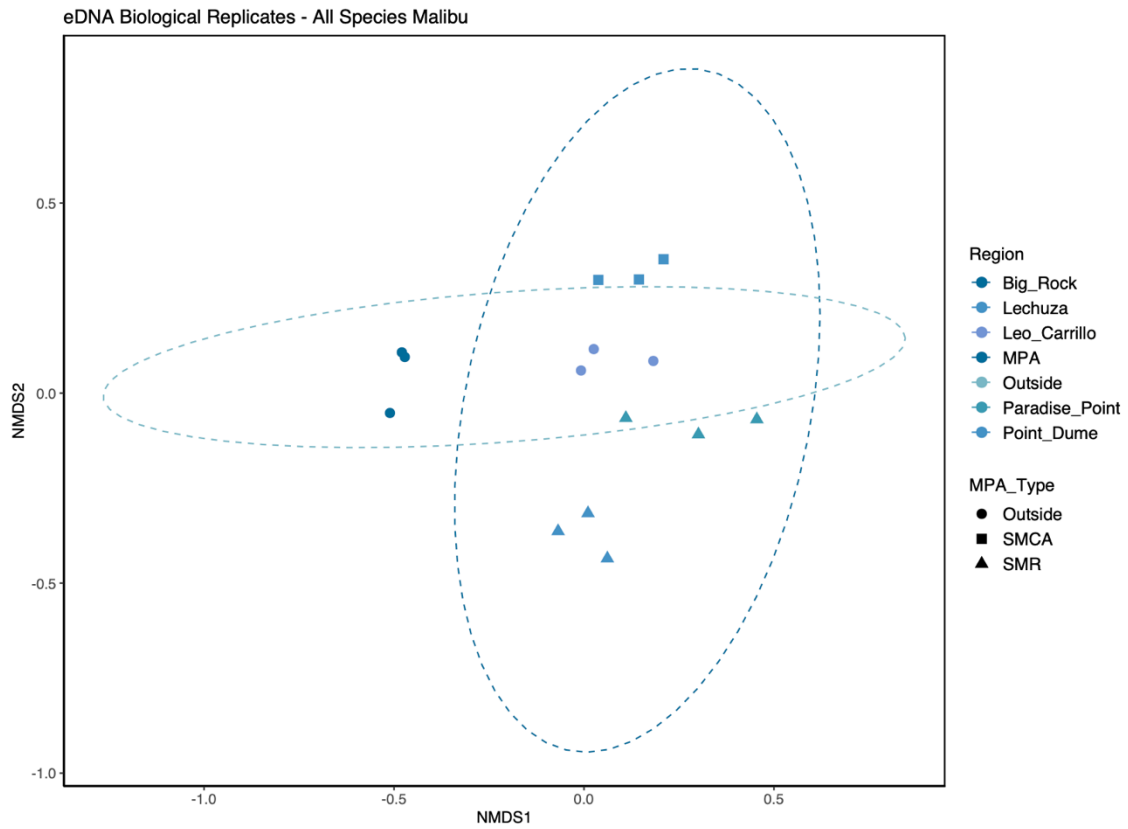


### Supplemental Figure 3-S4. NMDS of Bray-Curtis Dissimilarities between eDNA

Biological Replicates around South Point State Marine Reserve, Santa Rosa Island Using All Species Observed by eDNA. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.189).



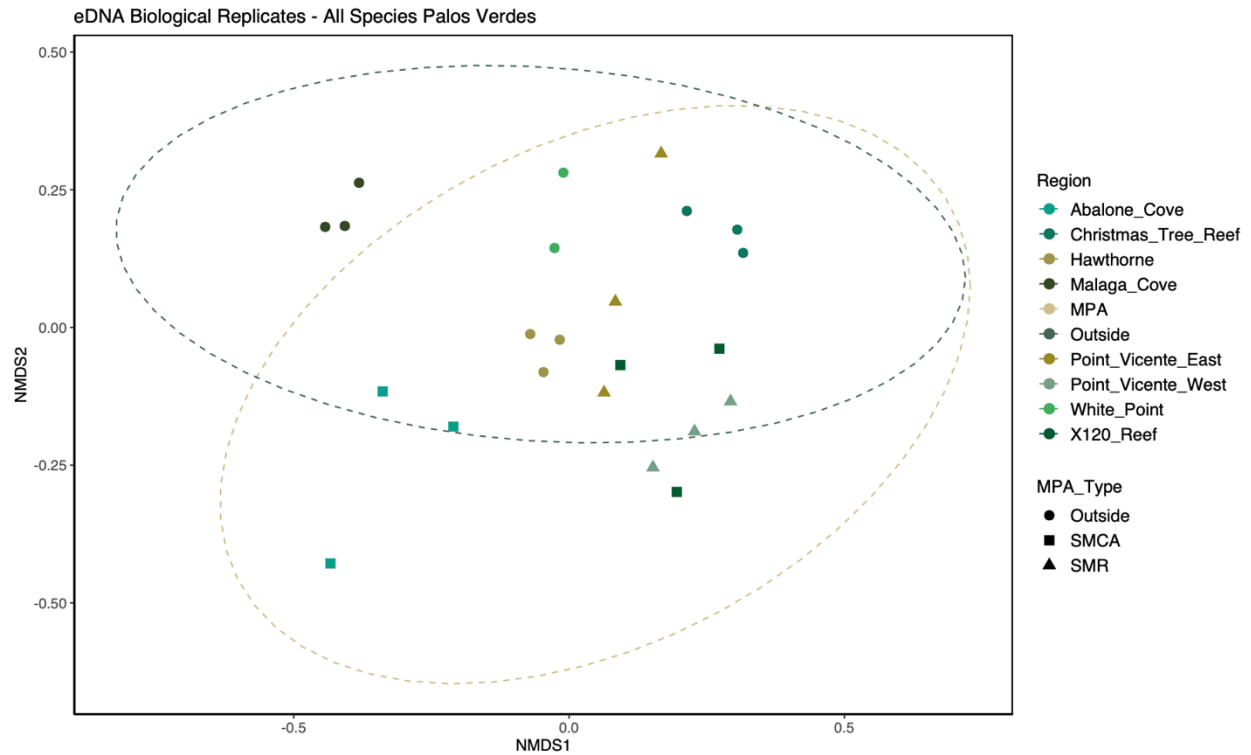
**Supplemental Figure 3-S5.** NMDS of Bray-Curtis Dissimilarities between eDNA Biological Replicates at Malibu Using All Species Observed by eDNA. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.115).



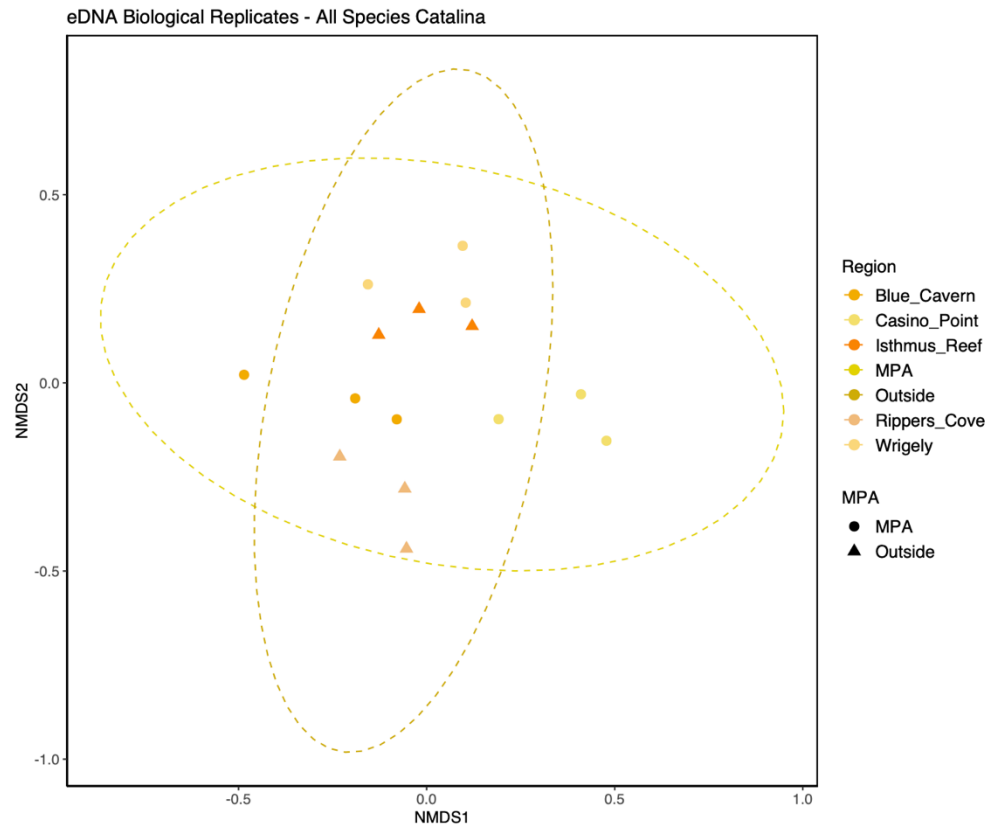


**Supplemental Figure 3-S6.** NMDS of Bray-Curtis Dissimilarities between eDNA

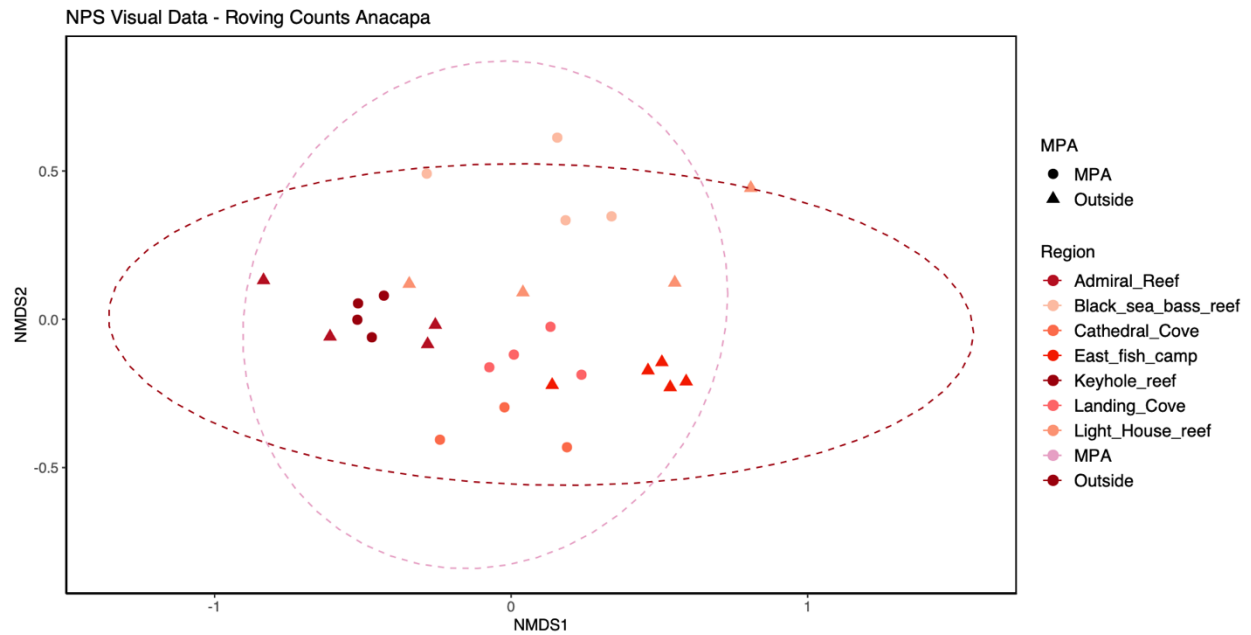
Biological Replicates at Palos Verdes Using All Species Observed by eDNA. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.169).



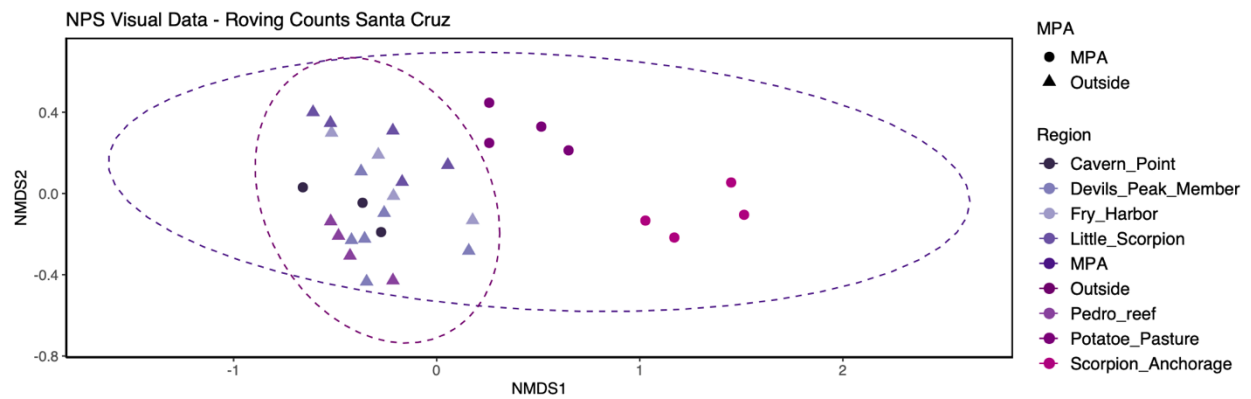
**Supplemental Figure 3-S7.** NMDS of Bray-Curtis Dissimilarities between eDNA Biological Replicates at Catalina Island Using All Species Observed by eDNA. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.159).



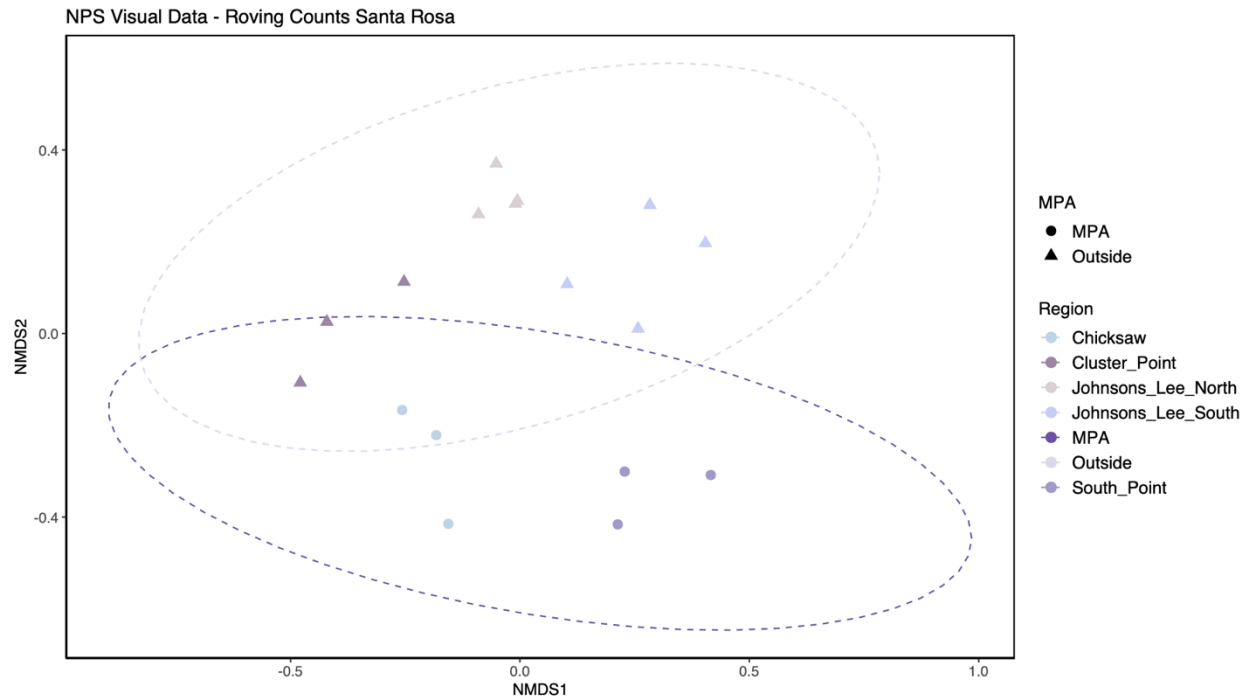
**Supplemental Figure 3-S8.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program Roving Diver Fish Count Surveys at Anacapa Island using Count Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.111).



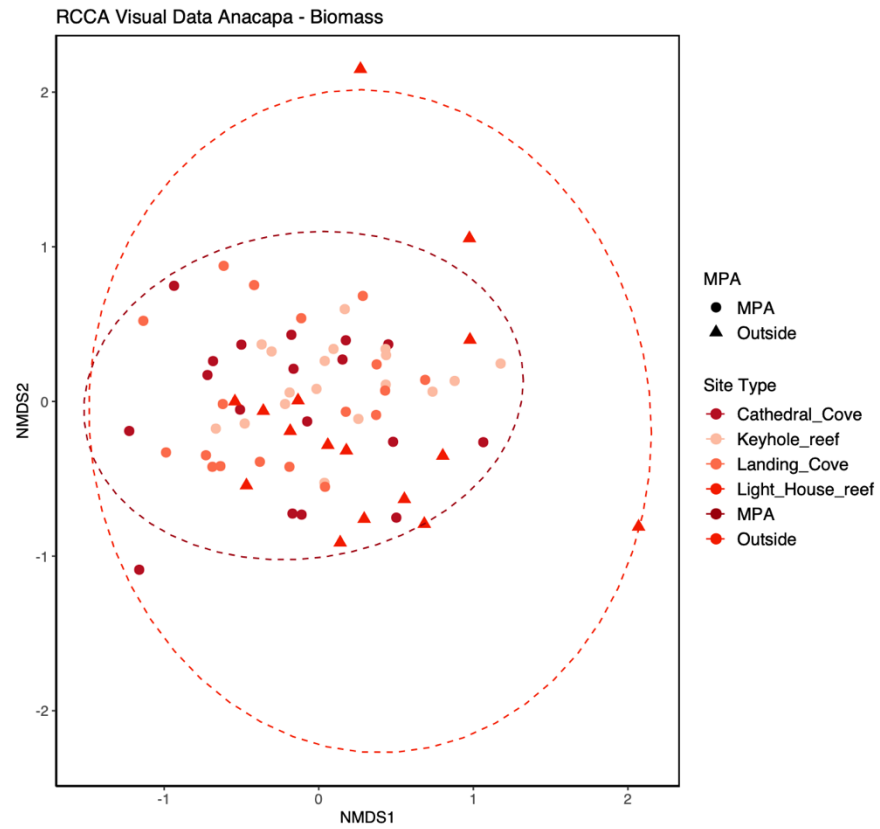
**Supplemental Figure 3-S9.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program Roving Diver Fish Count Surveys at Santa Cruz Island using Count Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.089).



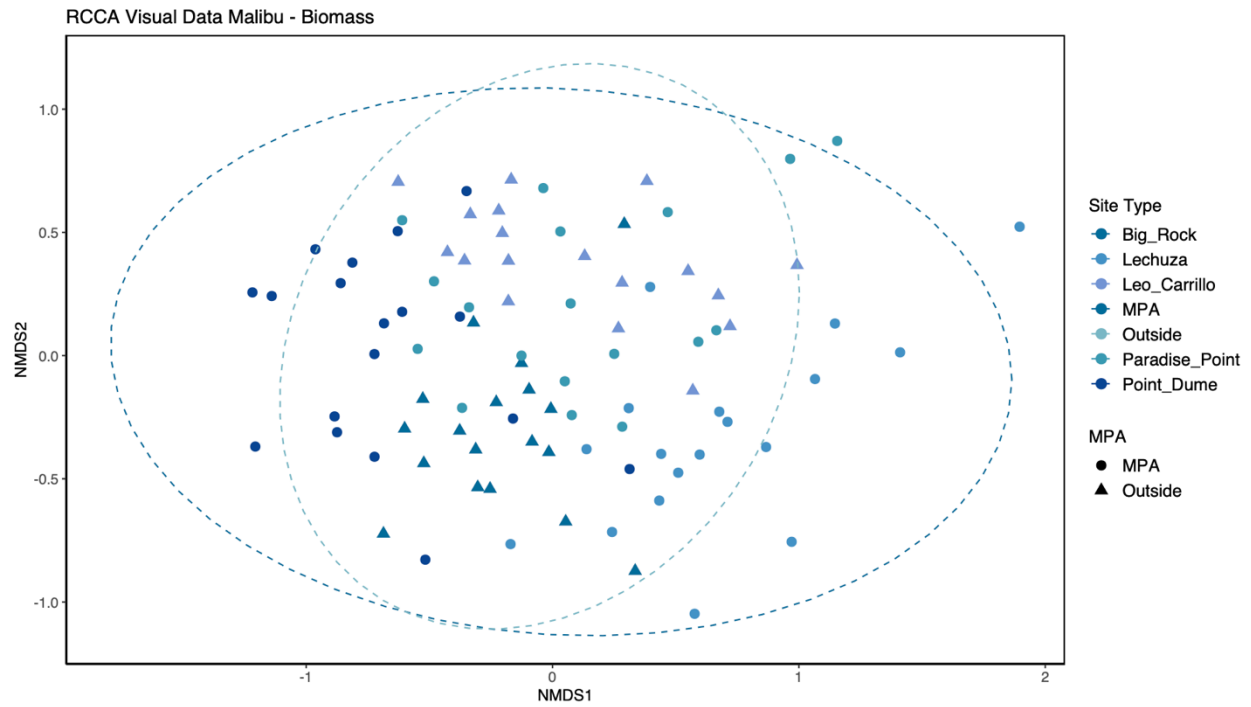
**Supplemental Figure 3-S10.** NMDS of Bray-Curtis Dissimilarities between Kelp Forest Monitoring Program Roving Diver Fish Count Surveys at Santa Rosa Island using Count Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.167).



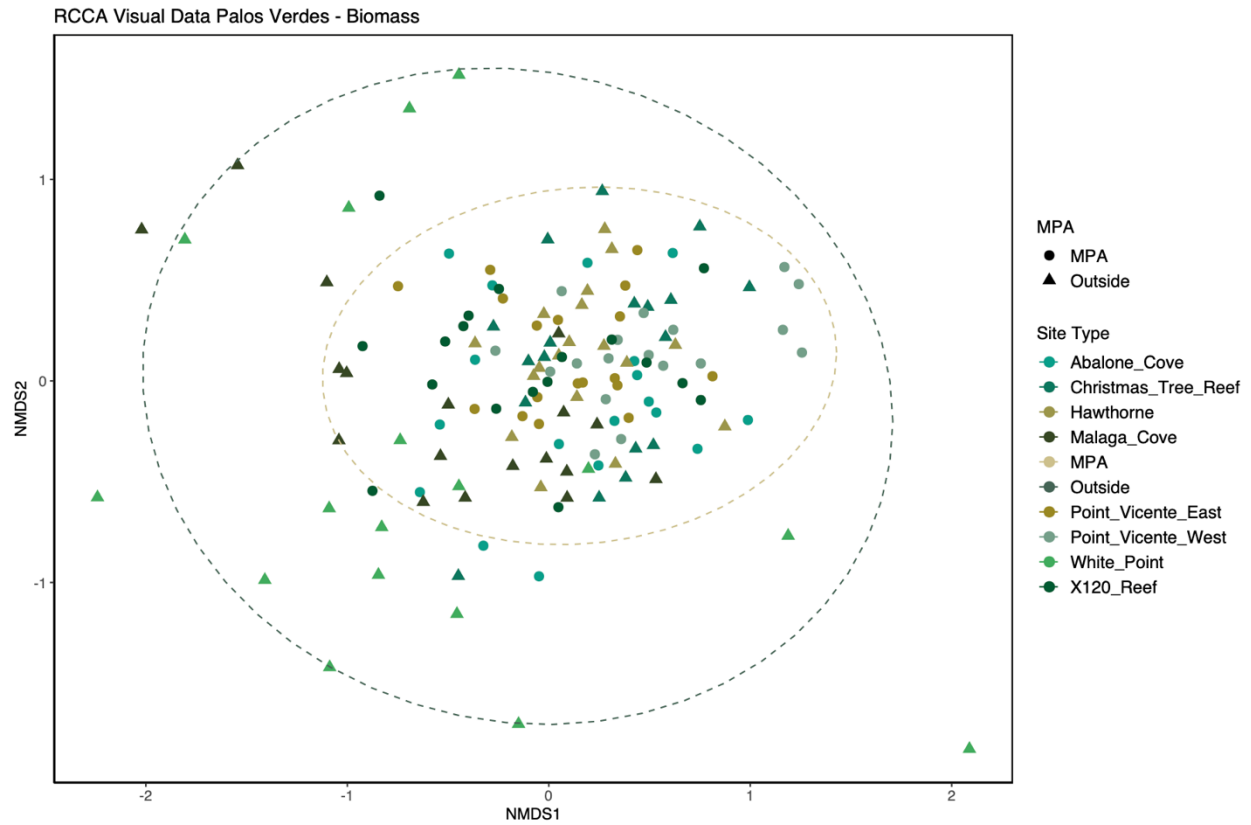
**Supplemental Figure 3-S11.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Surveys at Anacapa Island using Biomass Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.22).



**Supplemental Figure 3-S12.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Surveys at Malibu using Biomass Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.224).

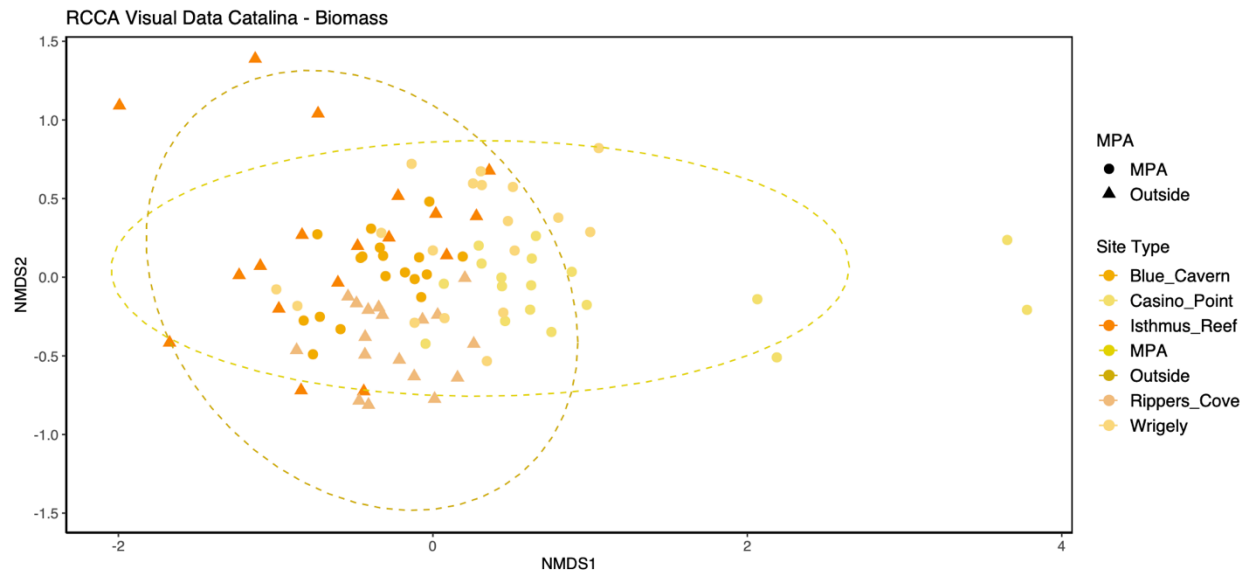


**Supplemental Figure 3-S13.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Surveys at Palos Verdes using Biomass Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.215).

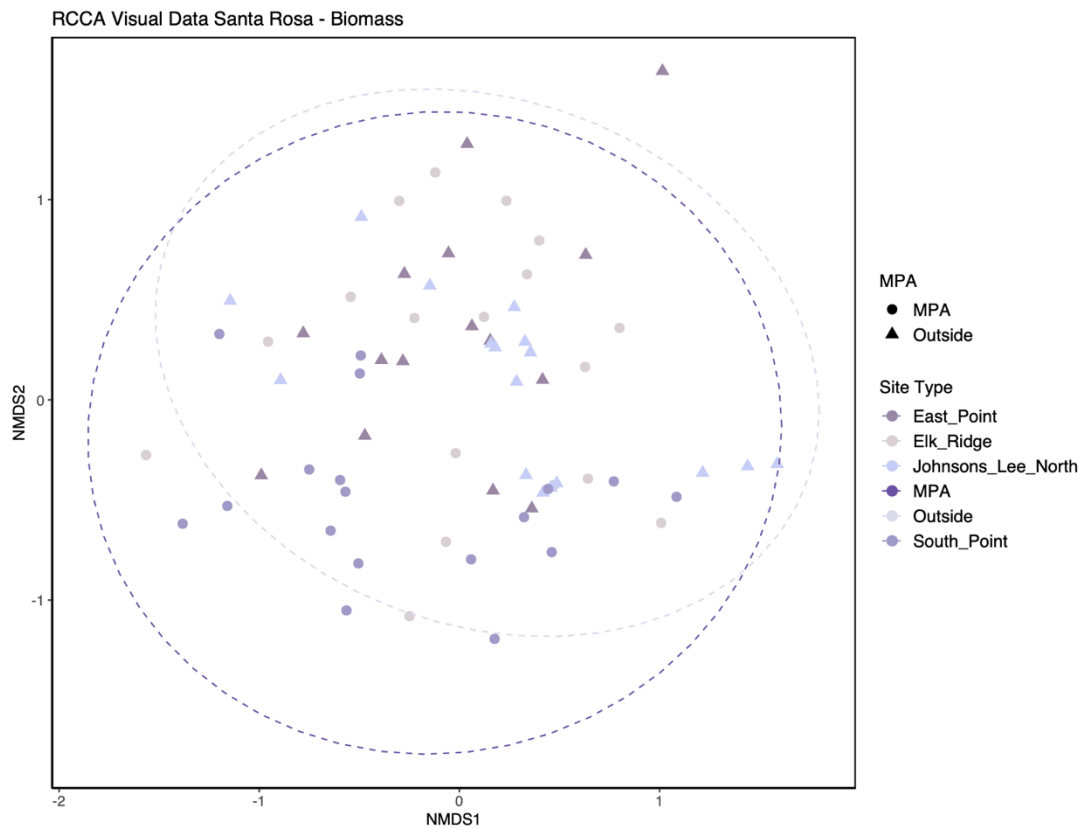




**Supplemental Figure 3-S14.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Surveys at Catalina Island using Biomass Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.147).



**Supplemental Figure 3-S15.** NMDS of Bray-Curtis Dissimilarities between Reef Check Visual Surveys at Santa Rosa Island using Biomass Data. Site (color) and MPA type (shape) are similar to each other (NMDS, Stress =0.206).



## APPENDIX D – SUPPLEMENTAL CHAPTER 2

### Supplemental Methods

#### *Study Sites*

We conducted eDNA and dip net surveys at three locations in San Diego County, California, USA in winter of 2017: 1) the Proctor Valley Vernal Pool Habitat Restoration Project, 2) Rancho Jamual, and 3) Otay Mesa. We selected sample sites from 10 vernal pools known to previously contain either *Branchinecta sandiegonensis* or *B. lindahli* (The Chapparal Lands Conservancy 2017a, 2017b) (Figure S2-1). At the Proctor Valley Vernal Pool Habitat Restoration Project site, we collected water samples from 8 pools (VP 1-8). These pools are deeper and retain water longer than other pools in Proctor Valley and were previously reported to be inhabited exclusively by *B. sandiegonensis* (The Chapparal Lands Conservancy 2017a, 2017b). At Otay Mesa, we surveyed a pool in a nearby urban development, VP-9, known to only have *B. lindahli* (The Chapparal Lands Conservancy 2017b). Lastly, at Rancho Jamual, we sampled one vernal pool (VP-10) known for its abundance of *B. sandiegonensis* (The Chapparal Lands Conservancy 2017a, 2017b).

We sampled most vernal pools three times. We took the first sample one week after the first significant winter rainfall that created the pools (The Chapparal Lands Conservancy 2017a). We took the second sample 1 month after ponding, and the third sample 2 months after ponding (See Supplemental Table S2-S1). We sampled multiple time points of the vernal pool hydroperiod to provide data across the fairy shrimp life cycle including immature larval stages to sexually active adults to senescence (Hildrew 1985). We subsequently refer to these sampling events as Time 1 (January 23, 2017), Time 2 (February 13, 2017), and Time 3 (March 7, 2017).

We only sampled VP-9 on the Otay Mesa at the third sampling point because we were notified that the pool had adult *B. lindahli* present after the Time 2 eDNA water sampling. In addition, we did not sample pool VP-8 at Time 2 as it was inaccessible. Because *B. sandiegonensis* is a federally endangered species and the sensitive nature of its habitat, we have chosen not to publish precise geographic coordinates for the study sites. Researchers interested in these data should contact the corresponding author.

#### *eDNA Field Collection and Filtration*

At vernal pools VP 1-8 and VP-10 we collected three replicate water samples in 1L Nalgene bottles attached to a telescoping pole with a hose clamp (Figure S2-1). In order to account for potential eDNA spatial heterogeneity, we sampled each pool at the center, median, and edge. The center sample was located as close to equidistant from the pool as possible, the edge sample was located at the closest proximity to the edge of the pool in which water could still be sampled, and the median sample was located between the center and edge samples (Supplemental Table S2-S1). Here in we refer to these three samples as replicates. At VP-9, we only sampled 1L due to the pools' small size (<1m diameter) and concerns for collecting *Branchinecta* species. To ensure that no animals were captured during eDNA water sampling, a 240  $\mu$ m net was used to exclude all animals from entering the Nalgene bottle. All captured particulates and organisms were returned to the pool.

To prevent cross contamination among samples, we initially sterilized all bottles and 240  $\mu\text{m}$  nets with a 10% bleach bath for 1 hour and rinsed three times with MilliQ nano-pure DNA free water (MilliporeSigma Corp., Burlington, MA, USA), and then placed each in a UV chamber for 15 minutes. In addition, to prevent cross contamination among pools, we sterilized the tip of the pole, the bottle clamp assembly and any part of the pole came into contact with pool water by soaking in 30% bleach for 15 minutes followed by rinsing with Nano-pure water. Each sampling day, we filled one 1L Nalgene bottle in the field with MilliQ Nano-pure DNA free water to serve as a negative control. We filtered eDNA from the water samples either through gravity filtration or manually, using a 50 mL syringe to push water through a 0.22  $\mu\text{m}$  Sterivex filter cartridge (MilliporeSigma Corp.) (Curd et al. 2019). Following filtration, we put the filter cartridge on ice, and then transferred them to the lab where they were stored at  $-20^{\circ}\text{C}$  until extraction. Due to the turbidity of the vernal pools, the entire 1L water sample collected could not be filtered. We filtered samples until the cartridge clogged and no additional water would pass through the filter. Sample volume ranged from 450 ml to as little as 50 ml. The total volume filtered for each sample is reported in Supplemental Table S2-S1.

#### *Reference DNA Collection and Sequencing*

To ensure our metabarcoding reference library included both *B. sandiegonensis* and *B. lindahli*, we generated DNA sequences of 10 individuals of *B. sandiegonensis* and 9 individuals of *B. lindahli* confirmed through morphological analysis. We isolated genomic DNA from specimens using either the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol or the AutoGenprep 965 using the AutoGen standard Mouse Tail (Animal Tissue) protocol (AutoGen Inc., Holliston, MA, USA). We conducted PCR amplification using the 16Sar and 16Sbr primers to target the *16S* rDNA gene (Palumbi 1991)

and amplified according to the protocols described in (Wetzer et al. 2018). We then purified PCR products using ExoSAP-IT (Aymetrix) and sequenced in both directions using BigDye (Applied Biosystems, Foster City, CA, USA) chemistry at the Smithsonian Lab Core Sequencing Facility (Washington, DC, USA). We trimmed and aligned forward and reverse sequence and assembled them into contigs in Geneious version 8.1.9; (<http://www.geneious.com>, Kearse *et al.* 2012) and accessioned into GenBank (Supplemental Table S2-S2).

#### *eDNA Filter Extraction*

In the laboratory, we extracted eDNA from filter cartridges within one week of collection using a modified DNeasy Tissue and Blood Qiagen Kit, adding the proteinase K and ATL buffer reagent the sterile filter cartridge (Spens et al. 2017, Curd et al. 2019). We then stored extracted DNA at -20°C before PCR.

#### *Contamination Precautions*

As false positives may result from contamination during eDNA collection as well as during DNA extraction and amplification, we employed multiple steps to minimize contamination and generation of false positives. Prior to water sample collection in the field, we sterilized all containers, supplies, and work surfaces with at least 10% bleach solution and always wore gloves to minimize the risk of contamination. In addition, we performed all DNA extractions in a PCR-free laboratory area, and all PCR preparations in an AirClean 600 PCR Workstation (AirClean Systems, Creedmoor, NC, USA) located in a clean room at UCLA dedicated to PCR/qPCR preparations. Prior to use, we sterilized the AirClean 600 PCR Workstation and pipettes before and after use with 10-30% bleach followed by a 30 min ultraviolet light (UV) treatment. Filtered pipette tips were used for all pre-PCR protocols. Lastly,

to test for any possible contamination, we implemented negative controls at each step, including field collections, DNA extraction, and PCR.

### *Library Preparation*

We amplified approximately 114-140bp of mitochondrial *16S* rDNA, using metazoan primers “16s\_Metazoa\_fwd” and “16s\_Metazoa\_rev” (Kelly et al. 2016) modified using Nextera adapter (Illumina, San Diego, CA, USA) sequences (Curd et al. 2019). We chose to use the *16S* rDNA metabarcoding primer because preliminary analyses using the Leray *COI* metabarcoding primer set did not resolve *Branchinecta* classification to species level (Leray et al. 2013; Data not shown). We performed PCR amplification in triplicate using a 25 µL reaction mixture containing 12.5 µL QIAGEN Multiplex Taq PCR 2x Master Mix, 6.5 µL of dH<sub>2</sub>O, 2.5 µL of each primer (2 µmol/L), and 1 µL template DNA. Thermocycler parameters employed a touchdown program: initial denaturation at 95°C for 15 min, 13 cycles of denaturation at 94°C for 30 sec, beginning annealing at 69.5°C for 39 sec (temperature was decreased by 1.5°C every cycle until 50°C was reached), and extension at 72°C for 1 min. Thirty five additional cycles were carried out at an annealing temperature of 50°C, followed by a final extension at 72°C for 10 min. For negative PCR controls, we used molecular grade water in place of DNA extractions, and positive controls used extracted DNA from *Gonodactylus childi*, a South Pacific marine stomatopod. We used this species because it does not occur in the study area, and therefore sequences from this species would unambiguously indicate contamination via post-PCR processes or index-hopping during sequencing. To confirm successful PCRs and product size, we electrophoresed all PCR products on 2% agarose gels.

We pooled triplicate PCR reactions using 5µL volume from each PCR, and then pooled PCR samples were cleaned using Serapure magnetic bead protocol (Faircloth and Glenn 2014).

We quantified bead-cleaned samples with the Quant-iT™ broad range dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) on a Victor3 plate reader (Perkin Elmer Waltham, MA, USA). We indexed the sample libraries using unique combinations of the Nextera Index A and D Kit (Illumina, San Diego, CA, USA) and KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Sigma Aldrich, St. Louis, MO, USA) (Curd et al. 2019). Indexing was performed with a second PCR using a 25 µL reaction mixture containing 12.5 µL of Kapa HiFi HotStart Ready mix, 0.625 µL of primer i7, 0.625 µL of primer i5, and 10 ng of template DNA. Index thermocycling parameters were: denaturation at 95°C for 5 min, 5 cycles of denaturation at 98°C for 20 sec, annealing at 56°C for 30 sec, extension at 72°C for 3 min, followed by a final extension at 72°C for 5 min. To confirm successful PCR and correct product size, we electrophoresed PCR products on 2% agarose gels. We then bead cleaned and quantified DNA concentration, as described above so that we could pool samples so as to have equal copy number of each three barcodes. In order to ensure adequate read depth across all samples, we sequenced the same eDNA library on two separate MiSeq runs with Reagent Kit V3 with 30% PhiX added. Sequencing was conducted at Technology Center for Genomics & Bioinformatics (University of California–Los Angeles, CA, USA).

### *Bioinformatics*

We processed the resulting sequence libraries separately using the *Anacapa Toolkit*, which performs quality control, amplicon sequence variant (ASV) parsing, and taxonomic assignment using user generated custom reference databases (Curd et al. 2019). We employed default parameters except for using a 70% minimum length and 70% mismatch for Bayesian Lowest Common Ancestor taxonomic classification. To assign taxonomy to ASVs, we used the *Anacapa Toolkit CRUX*-generated 16S rDNA reference databases (Curd et al. 2019) as generated



February 2019, and supplemented this database manually by adding the *Branchinecta* species barcode sequences generated specifically for this study.

### *Decontamination*

For all downstream decontamination and data analyses, we transferred the resulting species community tables into *R* using *ranacapa* (Kandlikar et al. 2018). We followed the multi-step decontamination protocol as outlined in Kelly et al. (2018).

#### Step 1: Estimation of Index Hopping

All samples were pooled into a final library and sequenced on a single MiSeq run. Each sample is identified by two sets of molecular barcodes in a unique combination. However, recent evidence has found that there is the potential for indexes to hop from one amplicon to another, leading to the incorrect sample assignment during demultiplexing (Costello et al. 2018). To estimate the frequency of index hopping we included a positive control of a non-native stomatopod taxa which we know will not be found in our eDNA samples (Kelly *et al.* 2018). Index hopping will lead to environmental sequences occurring in the positive control and vice versa. To estimate the frequency of index hopping, we modeled the composition of environmental sequences observed on the positive controls and subtracted these sequences from the environmental samples run. For example, if 12 reads of Garibaldi (*Hypsypops rubicundus*) are found in the positive control, we subtract 12 reads from the read counts of Garibaldi found in all environmental samples.

#### Step 2: Remove Contamination from Positive and Negative Controls

Here we remove ASVs that occur in positive and negative controls more frequently than environmental samples. For each ASV we determine whether the source is from a control or

environmental sample. For each ASV we calculate the maximum proportion, mean proportion, total number of reads, and prevalence of reads in both positive and environmental samples. We then use the following decision tree: If all four statistics are higher in one of the groups, we label it either control influence or environmental sequence; if there are conflicting results, we only remove ASVs in which the maximum abundance occurred in the positive control, otherwise we keep the ASVs.

### Step 3: Site Occupancy Modelling

We use site-occupancy modeling to determine whether the presence of an ASV is a true reflection of biological reality (i.e. *Branchinecta* sp. was present) or the result of a PCR artifact. This is challenging because it requires discriminating between PCR artifacts from rare (but real) organisms. Site-occupancy modeling provides a robust statistical framework to determine if the presence pattern of an ASV reflects a PCR artifact or rare organism (Royle and Link 2006, Schmidt et al. 2013). The binomial model yields the likelihood that an ASV (or taxon) detected is truly present in the sample. The model, implemented in Stan for R ( <https://mc-stan.org/> ; Team 2014), depends upon three parameters: 1) the commonness of an ASV (or taxon) in the dataset (denoted  $\Psi$ ), 2) the probability of a detection given that the ASV (or taxon) is truly present (true positive detection; denoted  $P_{11}$ ), and 3) the probability of a detection given that the ASV (or taxon) was not truly present (false positive; denoted  $P_{10}$ ). The probability occurrence function used was the following:

$$\text{Probability of Occurrence} = (\Psi \times (P_{11}^N) \times (1-P_{11})^{(K-N)}) / ((\Psi \times (P_{11}^N) \times (1-P_{11})^{(K-N)}) + ((1-\Psi) \times (P_{10}^N) \times ((1-P_{10})^{(K-N)})))$$

Where  $K$  is the number of samples taken within a site and  $N$  is the number of ASV detections within a site.

We used reasonably informative priors for parameter estimations. First, we assume that our primers do a reasonably good job of detecting species, if the species are present. Thus, true positive probability ( $P_{11}$ ) was modeled with priors from a left-skewed beta distribution where  $\alpha = 6$  and  $\beta = 1$ . Occupancy probability ( $\Psi$ ) modeled with different informative priors for *B. sandiegonensis* and *B. lindahli* given their different distributions across all sampled vernal pools. Since *B. sandiegonensis* is common across all sampled vernal pools in this study, we used weak priors from a left-skewed beta distribution where  $\alpha = 1$  and  $\beta = 2$ . In contrast, given that *B. lindahli* is rare across all sampled pools in this study, we used stronger priors from a left-skewed beta distribution where  $\alpha = 1$  and  $\beta = 6$ . Lastly, we assume that the false-positive rate of detection is unlikely to approach the true-positive rate. Thus, false positive probability ( $P_{10}$ ) was modeled with priors from a left-skewed beta distribution where  $\alpha = 1$  and  $\beta = 100$ . Stan occupancy models are included in Supplemental Materials as attached text files.

For each species we calculated the number of detections out of the number of samples taken at each pool and each time point. The occurrence of a sequence for one species in a given replicate was treated as a detection at that site. Each pattern of occurrence for a given ASV within a given site was considered a case (e.g. 2 detections out of 3 replicates). We then summarized the number of occurrences of each case and ran each case through a separate occupancy model to reduce computational time. Each unique model was run 10 times in order to filter out cases in which the model converged into a local maximum. We then removed all ASVs which had a modeled site occupancy probability of less than 80%.

### *Traditional Dip Net Survey Specimen Collection and Quantification*

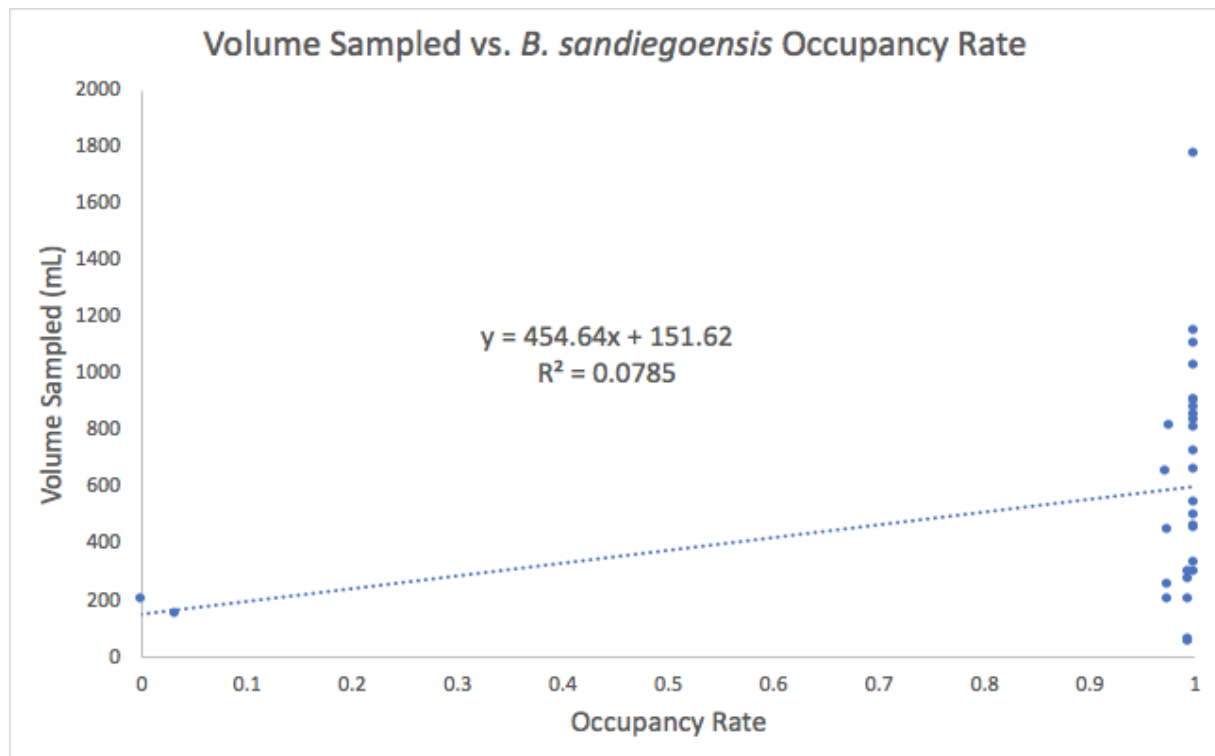
We simultaneously conducted traditional dip net surveys during eDNA surveys to compare the effectiveness of *Branchinecta* species detection for each method. All invertebrate samples were collected under USFWS permit number TE-221290-4. We conducted net sampling by sweeping a rectangular 240  $\mu$ m mesh net with a 13 cm x 15 cm opening along the full length of the vernal pool approximately 2-3 cm above the bottom. The whole catch was immediately fixed and preserved in 95% non-denatured ethanol see Supplemental Table S2-S3. To maximize DNA preservation, we transferred samples into fresh 95% non-denatured ethanol within 24 hours after collection. In the lab, we then morphologically sorted all adult *Branchinecta* species to species level (Supplemental Table S2-S3). Based on these results, we calculated site occupancy rates of dip net samples across all pools and at the landscape level according to Royle and Link (2006) as detailed below.

### **Supplemental Results**

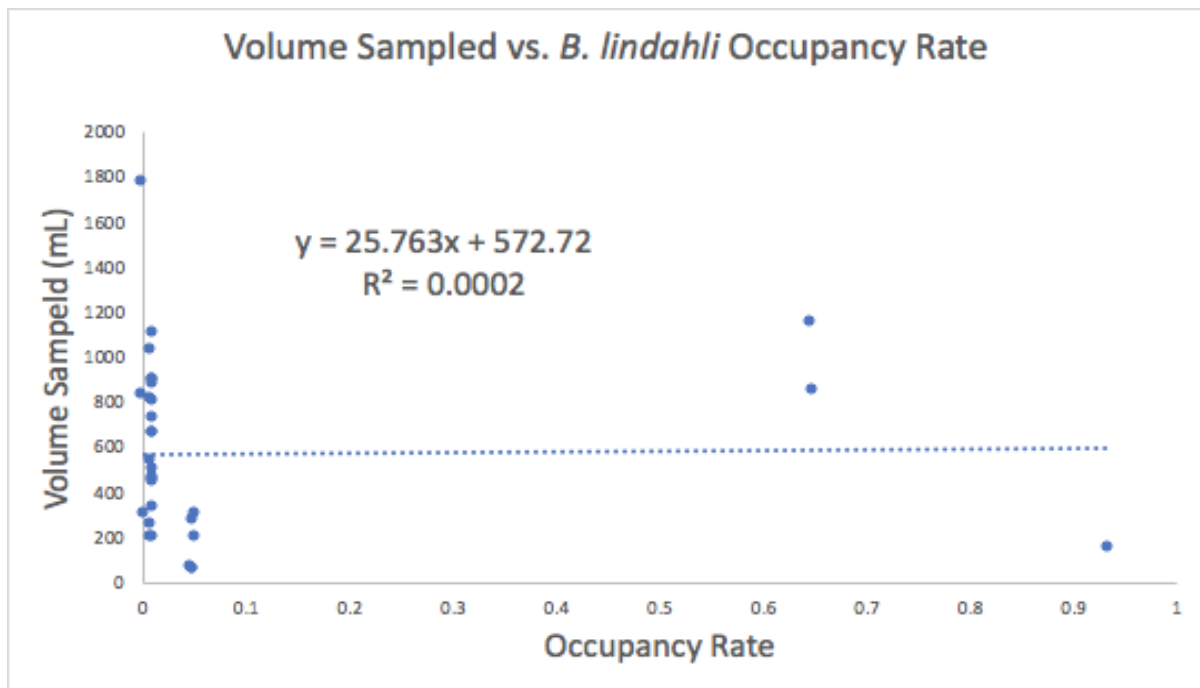
We fit a linear regression between total volume sampled from each vernal pool across all replicates against occupancy rates of *B. sandiegonensis* and *B. lindahli* in each vernal pool. For *B. sandiegonensis*, we found a weak correlation between volume sampled and occupancy rates ( $R^2$  0.08,  $p > 0.05$ ) (Supplemental Figure S2-S1). For *B. sandiegonensis*, we also found a weak correlation between volume sampled and occupancy rates ( $R^2$  0.08,  $p > 0.05$ ) (Supplemental Figure S2-S2). These results suggest that filtering samples until the filters clogged instead of maintaining constant volume did not have a significant negative affect on our sampling design.

**Supplemental Figure S2-S1. Volume Sampled vs. *B. sandiegonensis* Occupancy**

**Rate.** We found a weak relationship between the total volume of water sampled at each vernal pool and the occupancy rate of *B. sandiegonensis* ( $R^2 = 0.08$ ,  $p > 0.05$ ).



**Supplemental Figure S2-S2. Volume Sampled vs. *B. lindahli* Occupancy Rate.** We found a weak relationship between the total volume of water sampled at each vernal pool and the occupancy rate of *B. sandiegonensis* ( $R^2 = 0.0002$ ,  $p > 0.05$ ).



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